

BACTERIAL BIOSURFACTANT: CHARACTERIZATION, ANTIMICROBIAL AND METAL REMEDIATION PROPERTIES

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE
DEGREE OF**

MASTER OF SCIENCE IN LIFE SCIENCE

BY

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DECLARATION OF CANDIDATE

The work embodied in this report is an original investigation carried out by me, on the topic “Bacterial biosurfactant: characterization, antimicrobial and metal remediation properties”, for partial fulfillment of degree in master of Life Science, NIT Rourkela. No part of this thesis has been submitted to any other University or Institution to confer any Degree or Diploma.

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ACKNOWLEDGEMENT

This project is by far the most significant accomplishment in my life and it would not have been impossible without people who supported me and believed in my potential. I would like to extend my gratitude and sincere thanks to my honourable supervisor **Dr. Surajit Das**, Assistant Professor, and Department of Life Science. I sincerely thank for his exemplary guidance and encouragement. I express my sincere thanks to Dr Samir Kumar Patra (H.O.D) and all the faculty members of Department of Life Science, NIT Rourkela for showing sustained interest and providing help throughout the period of my work. I express my heartfelt gratitude to Miss Jaya Chakraborty (PhD scholar, Department of life science) for her constant guidance and support. I am also thankful to all the PhD scholars for their help.

I am also thankful to Mr. Rajesh Pattnaik, Department of Metals and Metallurgy for allowing me to avail the facility of FTIR and Scanning Electron Microscopy (SEM). I am also thankful to Department of Chemical engineering for allowing me to use tensiometer. I am also thankful to Department of Chemistry for allowing me to use Atomic Absorption Spectrophotometer.

I am genuinely appreciative of all my batchmates for their suggestions and moral support during my work.

Last, but not the least, I would thank the Almighty and my parents, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

Sneha Chakrabati

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ABSTRACT

Biosurfactants are amphiphilic compounds i.e., they contain both hydrophilic and hydrophobic moieties which partitions preferentially at the interfaces such as liquid/liquid, gas/liquid or solid/liquid interfaces. This facilitates properties like emulsification, foaming, detergency and dispersing. Their low toxicity and eco-friendly nature and the wide range of potential industrial applications in bioremediation, health care, food processing and oil industries makes them a highly useful group of chemical compounds. They are produced by a variety of microorganisms as extracellular compounds. Biosurfactants are superior to the chemical surfactants with respect to their biocompatibility, lower toxicity, higher biodegradability, higher stability, extreme stability in extreme temperature and pH. With the advent of time, this attribute is contributing its higher demand in the field of biotechnology. This study focus on the screening, production, extraction and purification of biosurfactant from bacteria isolated from petrochemical wastes and marine water and their chemical characteristics were elucidated. The antimicrobial activity of these biosurfactants was studied and their effect on lead remediation was also deliberated.

Key Words: Biosurfactant, surface tension, biocompatibility, bioremediation, petrochemical, lead

INTRODUCTION

Biosurfactants are amphiphilic compounds which are produced on living surfaces, mainly on surfaces of microorganisms or may also be secreted extracellularly and it contains both hydrophilic and hydrophobic moieties which reduce the surface and interfacial tension of the surface and interface respectively. Since biosurfactant and bioemulsifiers both exhibit emulsification properties, bioemulsifiers are frequently considered with biosurfactant, even though emulsifiers may not lower surface tension. A biosurfactant can have one of the following structures: glycolipids, mycolic acid, polysaccharide–lipid composite, lipoprotein/lipopeptide, phospholipid, or the microbial cell surface itself.

Significant attention has been given in the past to the synthesis of surface-active molecules from biological source because of their potential use in food-processing (Ramana and Karanth, 1989), oil industry, and pharmacology. Even though the type and quantity of the microbial surfactants produced depends mainly on the producer organism, factors like nitrogen and carbon, temperature, aeration and trace elements also affect their production by the organism.

Hydrophobic pollutants present inside petroleum hydrocarbons, and soil and water environment necessitate solubilisation before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Surfactants can raise the surface area of hydrophobic resources, such as pesticides in water and soil surroundings, thus increasing their water solubility. Hence, the existence of surfactants might increase microbial degradation of pollutants. The utilization of biosurfactants for the degradation of pesticides in soil and water environment has gained significance recently. The identification and characterization of biosurfactant produced by a variety of microorganisms have been broadly reviewed (Lin, 1996; Desai, 1987; Parkinson, 1985). Therefore, rather than recounting the several types of biosurfactants and their properties, this study specifies the production, characterization, surface tension reduction ability, antimicrobial activity of biosurfactant and its role in the hydrocarbon removal from environment and its efficacy in metal removal.

Microorganisms make use of a wide range of organic compounds as a source of carbon and energy for their growth. When the carbon source is in an insoluble form like a hydrocarbon (C_xH_y), microorganisms make possible their diffusion into the cell by producing a variety of substances, the biosurfactants. Some of the bacteria and yeasts excrete ionic surfactants which emulsify the C_xH_y substance in the growth medium. A few examples of this

group of biosurfactant are rhamnolipids that are produced by different *Pseudomonas sp.* (Guerra *et al.*, 1984; Hauser and Karnovsky, 1954; Guerra *et al.*, 1986; Hauser and Karnovsky, 1958; Burger *et al.*, 1963) or sophorolipids that are produced by several *Torulopsis sp.* (Cutler and Light, 1979, Cooper and Paddock, 1983). Some other microorganisms are able to change the structure of their cell wall, which are achieved by them by producing nonionic or lipopolysaccharides surfactants in their cell wall. Some examples of this group are: *Candida lipolytica* and *C. tropicalis* that produce cell wall-bound lipopolysaccharides when they are growing on n-alkanes (Osumi; *et al.*, 1975; Fukui and Tanaka, 1981); and *Rhodococcus erythropolis*, and various *Mycobacterium sp.* and *Arthrobacter sp.* which produce nonionic trehalose corynomycolates (Spencer 1979; Ristau and Wanger 1983; Kretschmer *et al.*, 1982; Rapp *et al.*, 1979; Kilburn and Takayama 1981; Suzuki *et al.*, 1968; Rosenberg *et al.*, 1979; Rubinowitz *et al.*, 1982). There are lipopolysaccharides, such as Emulsan, produced by *Acinetobacter sp.* (Rosenberg *et al.*, 1979; Rubinowitz, *et al.*, 1982), and lipoproteins such as Surfactin and Subtilisin, that are produced by *Bacillus subtilis* (Cooper *et al.*, 1981; Kakinuma *et al.*, 1969; Arima *et al.*, 1968). Other effectual biosurfactant are:

- i. Mycolates and Corynomycolates that are synthesized by *Rhodococcus sp.*, *Corynebacteria sp.*, *Mycobacteria sp.*, and *Nocardia sp.* (Cooper *et al.*, 1981; Kretshmer *et al.*, 1982; MacDonald *et al.*, 1981); and
- ii. Ornithinlipids that are synthesized by *Pseudomonas rubescens*, *Gluconobacter cerinus*, and *Thiobacillus ferrooxidans* (Knoche and Shively, 1972; Tahara *et al.*, 1976; Wilkinson and Galbraith, 1975).

Classification of biosurfactants

Unlike the chemically synthesized surfactants that are generally categorised on the basis of on the type of the polar group present, biosurfactants are in general classified chiefly by their chemical composition and microbial origin. Rosenberg and Ron (Rosenberg and Ron, 1999) suggested, biosurfactants could be divided into low molecular mass molecules that efficiently lower surface and interfacial tension, and large molecular- mass polymers, that are more efficient as emulsion-stabilizing agents. The main classes of low-mass surfactants are lipopeptides, glycolipids and phospholipids, whereas large-mass surfactants include polymeric and particulate surfactants. The majority biosurfactants are either anionic

or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic moiety can be a carbohydrate, phosphate, amino acid, or cyclic peptide (Nitschke and Coast, 2007) A concise discussion about each group of biosurfactant is given below (Table 1).

Table 1: Major biosurfactant classes and microorganisms involved (Karanth, *et al.*, 1999)

Surfactant class	Microorganism
Glycolipids Rhamnolipids Trehalose lipids Sphorolipids Mannosylerythritol lipids	<i>Pseudomonas aeruginosa</i> <i>Rhodococcus erithropolis</i> <i>Arthobacter</i> sp. <i>Candida bombicola</i> , <i>C. apicola</i> <i>C. antarctica</i>
Lipopeptides Surfactin/iturin/fengycin Viscosin Lichenysin Serrawettin	<i>Bacillus subtilis</i> <i>P. fluorescens</i> <i>B. licheniformis</i> <i>Serratia marcescens</i>
Phospholipids	<i>Acinetobacter</i> sp. <i>Corynebacterium lepus</i>
Surface-active antibiotics Gramicidin Polymixin Antibiotic TA	<i>Brevibacterium brevis</i> <i>B. polymyxa</i> <i>Myxococcus Xanthus</i>
Fatty acids or neutral lipids Corynomicolic acids	<i>Corynebacterium insidibasseosum</i>
Polymeric surfactants Emulsan Alasan Liposan Lipomanan	<i>Acinetobacter calcoaceticus</i> <i>A. radioresistens</i> <i>C. lipolytica</i> <i>C. tropicalis</i>
Particulate biosurfactants Vesicles Whole microbial cells	<i>A. calcoaceticus</i> <i>Cyanobacteria</i>

Glycolipids: Most of the biosurfactants are glycolipids. They are carbohydrates in grouping with long-chain aliphatic acids or hydroxyaliphatic acids. The connection is by means of either an ether or ester group. Among the glycolipids, the best known are rhamnolipids, sophorolipids and trehalolipids.

Rhamnolipids: The glycolipids, in which one or two molecules of rhamnose are connected to one or two molecules of β -hydroxydecanoic acid, are the most studied ones. The -OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the -OH group of the second acid is occupied in ester formation (Karanth *et al.*, 1999). The production of rhamnose which contains glycolipid was first studied in *Pseudomonas aeruginosa* by Jarvis and Johnson (Jarvis and Johnson, 1949). L-Rhamnosyl-L-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (Fig 1) and L-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate, referred to as rhamnolipids 1 and 2 respectively, are principal glycolipids synthesized by *P. aeruginosa* (Edwar, and Hayashi, 1965).

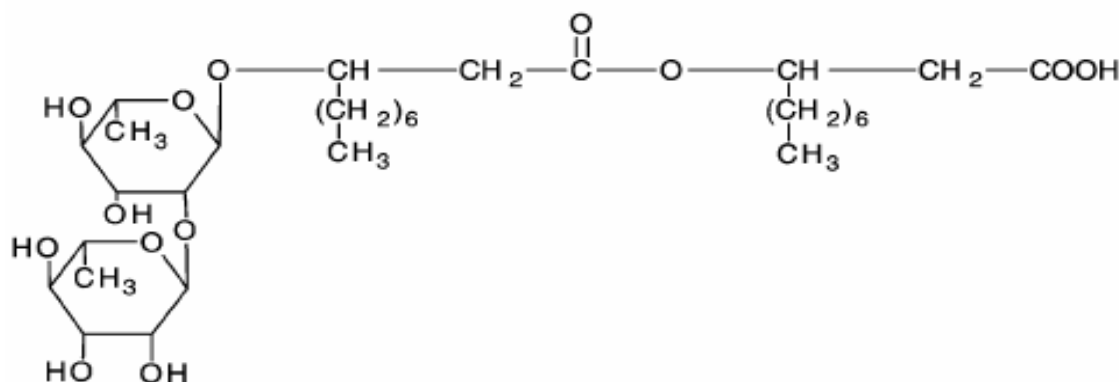


Fig 1: Structure of rhamnolipid.

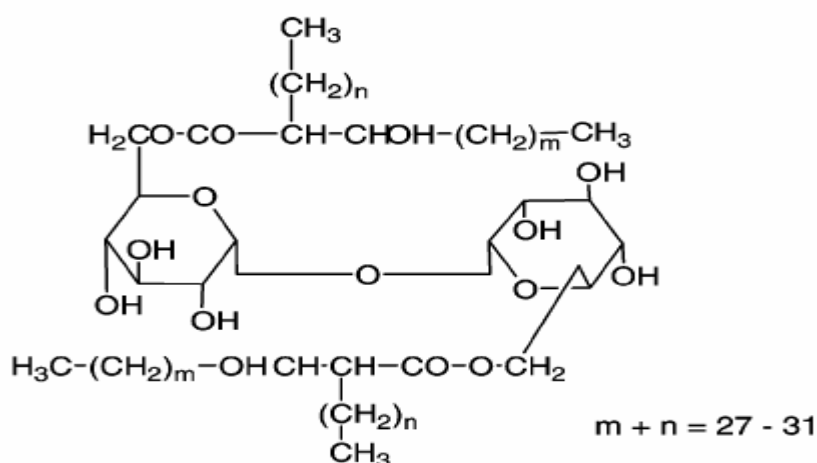


Fig 2: Structure of trehalose lipids.

Trehalolipids: Various structural types of microbial trehalolipid biosurfactants have been reported (Fig 2). Disaccharide trehalose linked at C-6 and C-6 to mycolic acid is related with most species of *Mycobacterium*, *Corynebacterium* and *Nocardia*. Mycolic acids are the long chain, α -branched and β -hydroxy fatty acids. Trehalolipids from diverse organisms vary in the size and structure of mycolic acid, the number of carbon atoms present and the extent of unsaturation (Asselineau and Asselineau, 1978). Trehalose lipids obtained from *Rhodococcus erythropolis* and *Arthrobacter sp.* reduced the surface tension and interfacial tension in culture broth (Kretschmer *et al.*, 1982).

Sophorolipids: These glycolipids that are synthesized mainly by yeast such as *Torulopsis bombicola* (Cooper and Paddock, 1984; Hommel *et al.*, 1987) (Fig 3), *T. petrophilum* and *T. apicola* consists of a dimeric carbohydrate sophorose attached to a long-chain hydroxyl fatty acid by a glycosidic linkage. Generally, sophorolipids are found as a mixture of free acid form and macrolactones. It is seen that the lactone form of the sophorolipid is essential, for various applications (Hu and Ju, 2001). These biosurfactants are a combination of at least six to nine varied hydrophobic sophorolipids.

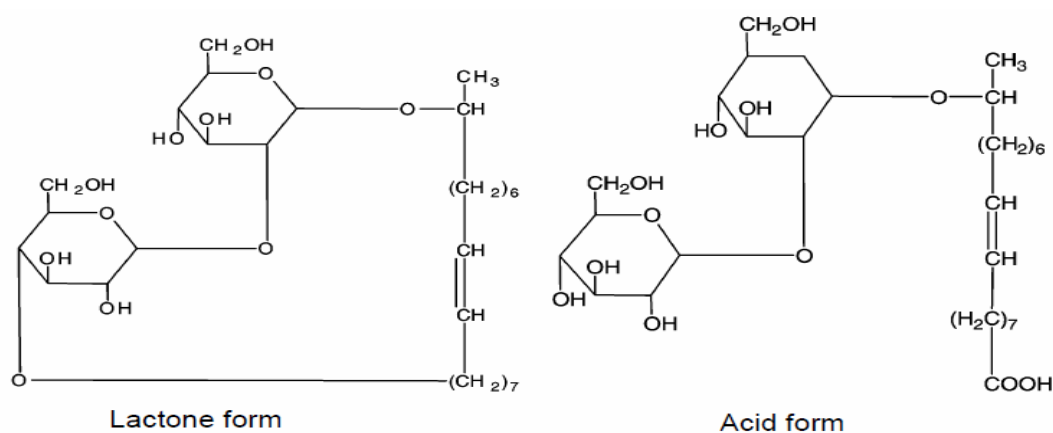


Fig 3: Structure of lactonized and free-acid forms of sophorolipids.

Lipopeptides and lipoproteins

A great number of cyclic lipopeptides, including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) are produced. These contain a lipid linked to a polypeptide chain.

Surfactin: The cyclic lipopeptide surfactin (Fig 4), synthesized by *Bacillus subtilis*, is one of the most potential biosurfactants. It is made up of a seven amino-acid ring structure joined to

a fatty-acid chain by means of lactone linkage. It reduces the surface tension from 72 to 27.9mN/m at a concentration as low as 0.005% (Arima *et al.*, 1968).

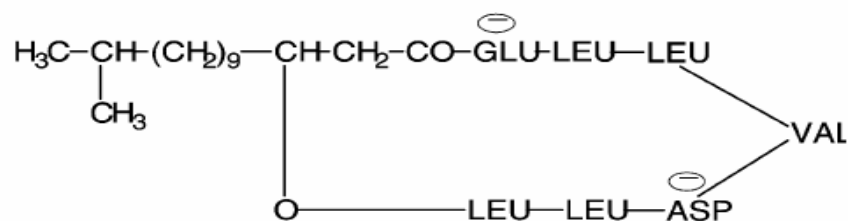


Fig 4: Structure of surfactin.

Lichenysin: *Bacillus licheniformis* synthesizes several biosurfactants that act synergistically and exhibit great temperature, salt and pH stability. They are also similar in their structural and physio-chemical properties to surfactin (McInerney *et al.*, 1990). The surfactants that are produced by *B. Licheniformis* are able to reduce the surface tension of water to 27mN/m and the interfacial tension between water and n-hexadecane to 0.36mN/m.

Fatty acids, phospholipids, and neutral lipids

Many bacteria and yeast synthesize large amounts of fatty acids and phospholipid surfactants during their growth on n-alkanes (Cirigliano and Carman, 1985). The hydrophilic and lipophilic balance (HLB) is directly proportional to the length of the hydrocarbon chain in their structures. In *Acinetobacter sp.*, phosphatidylethanolamine rich vesicles are synthesized (Kappeli and Finnerty, 1979) that form optically clear microemulsions of alkanes in water. Phosphatidylethanolamine synthesized by *R. Erythropolis* grown on n-alkane lowers the interfacial tension between hexadecane and water to less than 1 mN/m and a critical micelle concentration (CMC) of 30 mg/l (Kretschmer *et al.*, 1982).

Polymeric biosurfactants

The best-studied polymeric biosurfactants are alasan, liposan, lipomanan emulsan and some other polysaccharide-protein complexes. *Acinetobacter calcoaceticus* RAG-1 synthesizes an extracellular potent polyanionic amphipathic heteropolysaccharide bioemulsifier (Rosenberg *et al.*, 1979). Emulsan is an effective emulsifying agent for hydrocarbons in water (Zosim *et al.*, 1982), even at a concentration as low as 0.001 to 0.01%.

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein (Cirigliano and Carman, 1984).

Particulate biosurfactants

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion, which plays a very important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* sp. having a diameter of 20–50 nm and a buoyant density of 1.158 g/cm³, consists of protein, phospholipids and lipopolysaccharide (Kappeli and Finnerty, 1979).

Properties of biosurfactants

Biosurfactants are of increasing interest for commercial use because of the continually increasing spectrum of available substances. There are various advantages of biosurfactants compared to their chemically produced counterpart. The major distinctive features of biosurfactants and a brief description of each property are given below:

Surface and interface activity

A good surfactant can lower surface tension of water from 72 to 35 mN/m and the interfacial tension of water/hexadecane from 40 to 1 mN/m (Mulligan, 2005). Surfactin produced from *B. subtilis* are able to reduce the surface tension of water to 25 mN/m and interfacial tension of water/hexadecane to <1 mN/m (Cooper *et al.*, 1981). Rhamnolipids from *P. aeruginosa* decrease the surface tension of water to 26 mN/m and the interfacial tension of water/hexadecane to <1 mN/m (Hisatsuka *et al.*, 1971). The sophorolipids from *T. bombicola* reduce the surface tension to 33 mN/m and the interfacial tension to 5 mN/m (Cooper and Cavalero, 2003). In general, biosurfactants are more effective and efficient and their CMC is about 10–40 times lower than that of chemical surfactants, i.e. less amount surfactant is required to get a maximum decrease in surface tension (Desai and Banat, 1997).

Temperature, pH and ionic strength tolerance

Many biosurfactants and their surface activities are not affected by environmental conditions such as temperature and pH. McInerney *et al.*, (McInerney *et al.*, 1990) suggested that lichenysin produced by *B. licheniformis* was not affected by temperature (up to 50°C), pH (4.5–9.0) and by NaCl and Ca concentrations up to 50 and 25 g/l respectively. A lipopeptide produced by *B. subtilis* was stable after autoclaving (121°C/20 min) and after 6 months at –18°C; the surface activity did not change from pH 5 to 11 and NaCl concentrations up to 20% (Nitschke and Pastore, 1990).

Biodegradability

Unlike synthetic surfactants, microbial-produced compounds are easily degraded (Mohan *et al.*, 2006) and chiefly suited for the environmental applications such as bioremediation (Mulligan, 2005) and dispersion of oil spills.

Low toxicity

Very little data are available in the literature regarding the toxicity of biosurfactants. They are in general considered as low or non-toxic products and therefore are appropriate for pharmaceutical, food and cosmetic uses. A study suggested that a synthetic anionic surfactant (Corexit) displayed an LC50 (concentration lethal to 50% of test species) against *Photobacterium phosphoreum* ten times lower than rhamnolipids, signifying the larger toxicity of the chemically derived surfactant. By comparing the toxicity of six biosurfactants, four of the synthetic surfactants and two commercial dispersants, it was seen that most biosurfactants degraded quicker, except for for a synthetic sucrose-stearate that showed structure homology to glycolipids and was degraded more rapidly than the biogenic glycolipids. It was also studied that biosurfactants showed higher EC50 (effective concentration to decrease 50% of test population) values than synthetic dispersants (Poremba *et al.*, 1991). A biosurfactant from *P. aeruginosa* was compared to a synthetic surfactant that is widely used in the industry, regarding toxicity and mutagenic properties. Both assays indicated a higher level of toxicity and mutagenic effect of the chemically derived surfactant, whereas the biosurfactant was considered to be slightly non-toxic and nonmutagenic (Flasz *et al.*, 1998). Emulsion forming and emulsion breaking stable emulsions can be produced with a lifespan of months and years (Velikonja and Kosaric, 1993). Biosurfactants may stabilize (emulsifiers) or destabilize (de-emulsifiers) the emulsion. High molecular mass biosurfactants in are general better emulsifiers than low-molecular-mass biosurfactants. Sophorolipids produced from *T. bombicola* are able to reduce surface and interfacial tension, but are not good emulsifiers (Cooper and Cavaleiro, 2003). On the contrary, liposan do not reduce surface tension, but have been used productively to emulsify edible oils (Cirigliano and Carman, 1985). Polymeric surfactants have additional advantages because they coat droplets of oil, thus forming stable emulsions. This property is mainly useful for making oil/water emulsions for cosmetics and food.

Chemical diversity

The chemical diversity of naturally formed biosurfactants offers a wide selection of surface-active agents with properties closely related to specific applications.

Applications of biosurfactants

All surfactants are chemically synthesized. However, very recently, much attention has been given towards biosurfactants due to their broad range of functional properties and diverse synthetic capabilities of microorganisms. Most significant is their environmental acceptability, as they are easily biodegradable and have low toxicity than the synthetic surfactants. These unique natures of the biosurfactants allow their utilization and possible replacement of chemically synthesized surfactants in a large number of industrial operations. Furthermore, they are ecologically safe and can be applied in wastewater treatment and bioremediation. Some of the potential applications of biosurfactants in pollution and environmental control are microbial enhanced oil revival, hydrocarbon degradation in the soil environment and hexa-chloro cyclohexane degradation, removal of heavy-metal from contaminated soil and hydrocarbon in aquatic environment (Singh, *et al.*, 2007).

Potential food applications

Biosurfactants can be explored for several food-processing applications. They are:

Food-formulation ingredients:

Apart from their obvious role as agents that decrease surface and interfacial tension, thus facilitating the formation and stabilization of emulsions, the surfactants can have various other functions in food. For example, to control the aggregation of fat globules, stabilization of aerated systems, improvement of texture and shelf-life of products containing starch, modification of rheological properties of wheat dough and improvement of constancy and texture of fat-based products (Kachholz and Schlingmann, 1987). In bakery and ice-cream formulations biosurfactants act by controlling the consistency, slowing staling and solubilizing the flavour oils; they are also agents during cooking of fats and oil. Improvement in the stability of dough, volume, texture and conservation of bakery products is obtained by the addition of rhamnolipid surfactants (Van Haesendonck and Vanzeveren, 2004). The study also suggested the use of rhamnolipids to improve the properties of butter cream and frozen confectionery products. L-Rhamnose has substantial potential as a forerunner for flavouring. It is already used industrially as a precursor of high-quality flavour components like furaneol.

Antiadhesive agents:

A biofilm is described as a group of bacteria that have formed a colony on a surface. The biofilm not only consists of bacteria, but it also includes all the extracellular material produced at the surface and any material trapped within the formed matrix. Bacterial biofilms that are present in the food industry surfaces are potential sources of contamination that may lead to food spoilage and transmission of disease (Hood and Zottola, 1995). Thus controlling the adherence of microorganisms to food-contact surfaces is an essential step in providing safe and quality products to consumers.

The involvement of biosurfactants in microbial adhesion and detachment from surfaces has been investigated. A surfactant produced by *Streptococcus thermophilus* has been used for fouling control of heat-exchanger plates in pasteurizers, as it slows down the colonization of other thermophilicso utilized as fat stabilizers and antisattering strains of *Streptococcus* that are responsible for fouling. The treatment of stainless steel surfaces with a biosurfactant obtained from *Pseudomonas fluorescens* inhibits the attachment of *L. monocytogenes*. The bioconditioning of surfaces through the use of microbial surfactants has been suggested as a new strategy to reduce adhesion.

Therapeutic and biomedical applications and Antimicrobial activity:

Several biosurfactants have shown antimicrobial action against various bacteria, algae, fungi, and viruses. The lipopeptide iturin from *B. subtilis* showed strong antifungal activity (Besson *et al.*, 1976). Inactivation of enveloped virus such as herpes and retrovirus was observed with 80 mM of surfactin (Vollenbroich *et al.*, 1997). Rhamnolipids repressed the growth of harmful bloom algae, *Heterosigma akashivo* and *Protocentrum dentatum* at a concentration varying from 0.4 to 10.0 mg/l. A rhamnolipid mixture obtained from *P. aeruginosa* showed inhibitory activity against the bacteria *Escherichia coli*, *Micrococcus luteus* and *Alcaligenes faecalis* (32 mg/ml), *Serratia arcscens* and *Mycobacterium phlei* (16 mg/ml) and *Staphylococcus epidermidis* (8 mg/ml) and excellent antifungal properties against *Aspergillus niger* (16 mg/ml), *Chaetonium globosum*, *Enicillium crysogenum*, *Aureobasidium pullulans* (32 mg/ml) and the phytopathogenic *Botrytis cinerea* and *Rhizoctonia solani* (18 mg/ml) (Abalos, *et al.*, 2001). The rhamnolipids and sophorolipids were found to be effective antifungal agents against plant and seed pathogenic fungi. The mannosylerythritolcations lipid (MEL), a glycolipid surfactant from *Candida antartica*, has shown antimicrobial activity particularly against Gram-positive bacteria (Kitamoto *et al.*, 1993).

Anticancer activity:

The biological activities of seven microbial extracellular glycolipids, together with mannosylerythritol lipids-A, mannosylerythritol lipids-B, rhamnolipid, polyol lipid, sophorose lipid, etc. have been studied. All these glycolipids, except for rhamnolipid, were able to induce cell differentiation instead of cell proliferation in the human promyelocytic leukaemia cell line HL60. STL and MEL noticeably increased common differentiation characteristics in monocytes and granulocytes respectively. Exposure of B16 cells to MEL lead to the condensation of chromatin, DNA fragmentation and sub-G1 arrest (the sequence of events in apoptosis). This is the first evidence that growth retards, apoptosis and differentiation of the mouse malignant melanoma cells can be induced by glycolipids (Zhao, 1999). In addition, exposure of PC12 cells to MEL enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase, with resulting outcome of neurites and partial cellular differentiation (Wakamatsu, 2001) lipid (MEL), a glycolipid surfactant from *Candida antartica*, has shown antimicrobial activity particularly against Gram-positive bacteria (Kitamoto *et al.*, 1993).

Anti-human immunodeficiency virus and sperm immobilizing activity:

The increased incidence of Human Immunodeficiency Virus (HIV)/AIDS in women aged 15–49 years has identified the urgent need for a female-controlled, effective and safe vaginal topical microbicide. To overcome this challenge, sophorolipid synthesized by *C. bombicola* and its structural analogues have been studied for their spermicidal, anti-HIV and cytotoxic activities (Shah *et al.*, 2005). The sophorolipid diacetate ethyl ester derivative is the most potent spermicidal and virucidal agent of the series of sophorolipids studied. The virucidal activity against HIV and sperm-immobilizing activity against human semen are similar to those of nonoxynol. Nevertheless, it also induced sufficient vaginal cell toxicity to raise concerns about its applicability for long-term microbicidal contraception.

Agents for respiratory failure:

A deficiency of pulmonary surfactant which is a phospholipid protein complex is responsible for the failure of respiration in prematurely born infants. Isolation of the genes for protein molecules of this surfactant and cloning in bacteria has made possible its fermentative production for medical applications (Gautam and Tyagi, 2005).

Agents for the stimulation of skin fibroblast metabolism:

The use of sophorolipids in lactone form comprises a major part of diacetyl lactones as agents for stimulating skin dermal fibroblast cell metabolism and mainly, as agents for the stimulation of collagen neosynthesis, at a concentration of 0.01 ppm at 5% (p/p) of dry matter in formulation. This can be applied in cosmetology and also in dermatology. The purified lactone sophorolipid product is of importance in the formulation of dermis anti-ageing products because of its effect on the stimulation of cells of the dermis. By encouraging the production of new collagen fibres, purified lactone sophorolipids may be used both as a preventive measure against ageing of the skin and used in creams for the body, and in the body milks, lotions and gels that are used for the skin (Borzeix and Frederique, 2003).

Antiadhesive agents in surgicals:

Pre-treatment of silicone rubber with surfactant produced by *S. thermophilus* inhibited by 85% the adhesion of *C. Albicans* (Busscher *et al.*, 1997) whereas surfactants obtained from *L. fermentum* and *L. acidophilus* adsorbed on glass, reduced by 77% the number of adhering uropathogenic cells of *Enterococcus faecalis*. The biosurfactant obtained from *L. fermentum* inhibited *S. aureus* infection and adhered to surgical implants (Gan *et al.*, 2002). Surfactin decreased the amount of biofilm formation by *Salmonella typhimurium*, *S. enterica*, *Escherichia coli* and *Proteus mirabilis* in PVC plates and vinyl urethral catheters (Mireles *et al.*, 2001).

REVIEW OF LITERATURE

Rapid Industrialization is resulting in unprecedented environmental pollution and hazards. Pollution due to petroleum oil is even now a prevalent ecological hazard and hence microbial degradation of hydrocarbons remains as a topical issue as before. The most common feature of all the crude oil is their low water solubility and this poses special problems for those microorganisms capable of utilizing such water immiscible substrates as source of carbon as well as energy. The initial step in the process is the transport of the hydrocarbon from oil phase to the cell surface in some way so as to achieve effective cell surface contact and ultimately efficient transportation across cell membrane. Although a lot amount of work has been done in this area, the mechanism of transport of n-alkane to bacterial cell and its subsequent assimilation inside cells still remains unclear. Two types of hydrocarbon interaction during biodegradation have been described earlier by Kirschner (Kirschner, *et al.*, 1980): adhesion to oil, and a hypothesized pseudo solubilization in which the hydrocarbon degrading bacteria assimilate small droplets of emulsified oil. Reports are available in favour of the first theory where microbial cells attach to the surface of hydrocarbon drops much smaller than the cells and substrate uptake presumably takes place through diffusion or active transport at the point of contact (Kennedy *et al.*, 1975, Rosenberg and Rosenberg, 1981). The hypothetical emulsification and incorporation of submicron droplets of oil (Rosenberg, *et al.*, 1979, Reddy, *et al.*, 1982) is analogous to the pseudo solubilization of oil hypothesized by Kirschner. Similar kind of theory was explained by Singh and Desai (Singh, and Desai, 1986). They referred to two modes of initial interaction of hydrocarbon with the microbial cells:

- (a) Direct interaction of microorganisms with insoluble substrate (unmediated interaction) or
- (b) By the contact through a mediator (mediated interaction).

The scale tilts in the favour of the latter theory involving the role of mediators or extracellular solubilising factor. Several investigators have shown the presence of emulsifiers in the culture broth during the growth of microorganisms on hydrocarbons and its consequence, due to solubilisation or emulsification, on hydrocarbon uptake (Cameotra *et al.*, 1984, Southam *et al.*, 2001, Cubitto *et al.*, 2004). Biosurfactants are present in various pools inside cells: as intracellular molecules, extracellularly secreted products or as compounds located at the cell surface (Prabhu, and Phale, 2003). Together they have been known to enhance degradation by alteration in cell hydrophobicity and enhancement of dispersion of

water immiscible compounds (Zang and Miller, 1992; Zang and Miller, 1994; Patricia and Jean-Claude, 1999). *Pseudomonas aeruginosa* strains are known to produce rhamnolipid type of biosurfactant which is a mixture of mainly mono and dirhamnolipids. Application of rhamnolipids to pure cultures or in soil systems has been shown to enhance biodegradation of a number of hydrocarbons including hexadecane (Oberbremer *et al.*, 1990, Herman *et al.*, 1997, Herman *et al.*, 1997 Sandrin *et al.*, 2000, Maslin, and Maier, 2000, Noordman, *et al.*, 2002). Thus a clear correlation exists between surface active agent production and alkane utilization by the degrading organism.

Biodegradation of hydrocarbons in soil can also be efficiently enhanced by addition or in situ production of biosurfactants. It was generally observed that the time required for degradation, and in particular the adaptation time, for microbes was shortened. Studies with chemical surfactants showed that the degradation of phenanthrene by an unidentified isolate could be increased by a nonionic surfactant based on ethylene glycol. In hydrocarbon contaminated soil, the elimination of polycyclic aromatics from the crude oil Arabian light was due to wave action or to microbial degradation. The chemically synthesized surfactant Finasol OSR-5 doubled the initial content of aromatics and decreased the amount of aromatics removed after 6 months, whereas adding the biosurfactant trehalose-5, 5'-dicorynomycolates caused complete elimination within the period. Interesting data were also obtained when soil trays (about 20 % moisture) contaminated with polycyclic aromatic hydrocarbons (PAH) were incubated (at room temperature) for 22 days and more. Nutrients with sophorose lipids (added at time 0) were blended into the soil. It is evident that many PAH were significantly removed. Even though PAH are most resistant to biodegradation they were degraded to a considerable degree in soil to which sophorose lipids were added. This removal was, however, dependent upon particular PAH. On the basis of the experiments by Kosaric *et al.*, (Kosaric *et al.*, 1987), the following conclusions can be drawn:

- i. Addition of sophorose lipids caused a sharp drop of metholachlor concentration in the methanol extract from soil slurry bioreactors.
- ii. Addition of sophorose lipids enhanced biodegradation of 2, 4-DCP in the soil slurry reactors.
- iii. Naphthalene was significantly more eliminated in the soil slurry bioreactor in which sophorose lipids were present.
- iv. Some polycyclic aromatic hydrocarbons (PAH) were almost completely removed in 22 days, in the presence of sophorose lipids in trays containing soil, while some were resistant to biodegradation.

- v. There is selectivity in biodegradation of PAH-s in soil.

Studies on biosurfactant-assisted bioremediation were also reported by other researchers. *P. aeruginosa*, isolated from oil-polluted sea water, was able to break down hexadecane, heptadecane, octadecane and nonadecane in seawater by up to 47, 58, 73 and 60 %, respectively, after 28 days of incubation (Shafeeq *et al.*, 1989). Presence of biosurfactants in the culture medium was shown by tensiometric measurements.

Pseudomonas aeruginosa UG2 biosurfactant when added to the soil contaminated with a hydrocarbon mixture of hexadecane, tetradecane, 2-methylnaphthalene and pristane. Enhanced degradation of all hydrocarbons, except 2-methylnaphthalene, was observed after 2 months incubation period (Jain *et al.*, 1992).

In another experiment (Banat, 1995), contaminated soil was inoculated with *Pseudomonas* ML2 or *Acinetobacter haemoliticus* and hydrocarbon degradations were compared with the same soil to which an ML2 biosurfactant product was added. After 2 months of incubation, 39-71 % reduction of hydrocarbons was achieved by *A. haemoliticus*, where as the *Pseudomonas* ML2 showed 11-71 % reduction. The results suggested that using cell-free biosurfactants, the degradation by indigenous microorganisms in the soil was stimulated.

It was also reported that the ability of a biosurfactant from *Bacillus* sp. to release oil from oily sand at a concentration of 0.04 mg/mL (Eliseev, *et al.*, 1991). Biosurfactants have also been demonstrated to successfully solubilize and remove hydrocarbon pollutants from contaminated soil. Examples are biosurfactant - containing broths from *Rhodococcus* ST-5 (Abu-Ruwaida *et al.*, 1991) and from the thermophilic *Bacillus* AB-2 (Banat, 1993). Rhamnolipid biosurfactants from *Pseudomonas aeruginosa* were characterized for their ability to remove hydrocarbons from sandy-loam soil and silt-loam soil (Van Dyke, *et al.*, 1993).

Large scale field applications were also done by Kosaric. Many contaminated sites in Canada and the Middle East were bioremediated with biosurfactant addition to the culture medium. These sites represented soil and sand contaminated by heavy hydrocarbons, primarily of industrial origin. Bioremediation was accelerated when glycolipid biosurfactants were added to the nutrient which was applied to the soil. Machine-oil-contaminated soil has been shown to be remediated by microbial inoculation and by biosurfactant treatment. Successful bioremediation of oil-contaminated soil and groundwater from a US Army engineering plant, using natural surfactants produced by indigenous microorganisms, was demonstrated (Fry *et al.*, 1993).

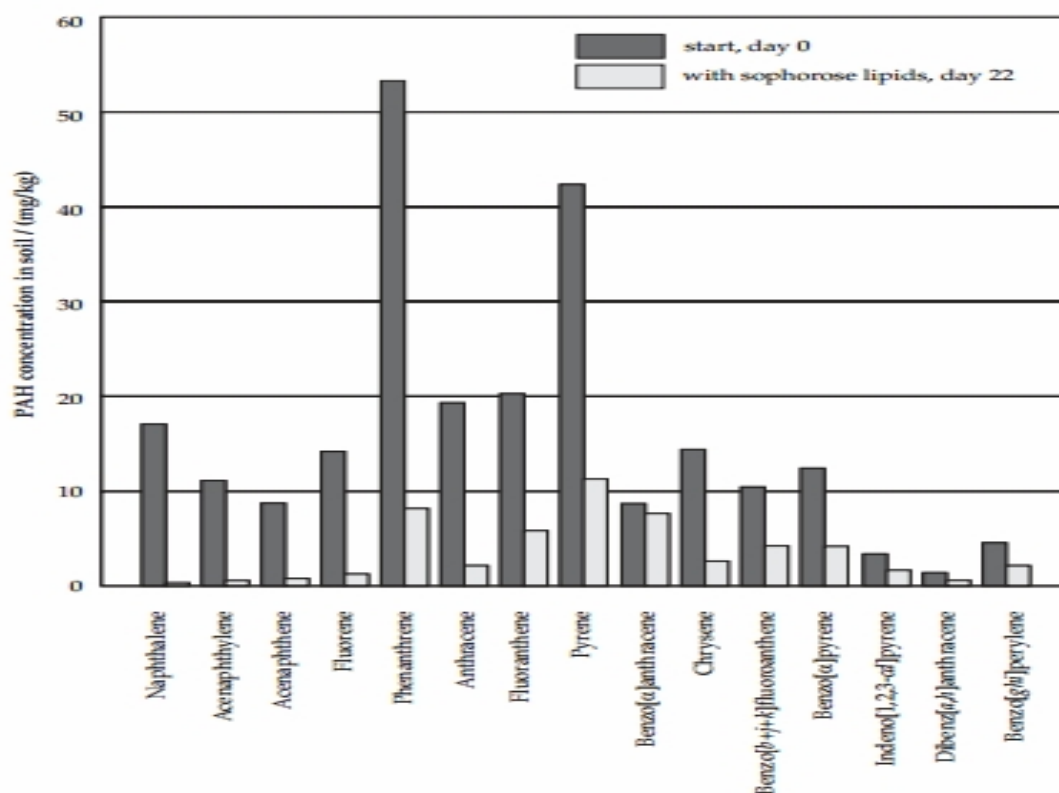


Fig 5: Biodegradation of PAHs in soil

AIMS AND OBJECTIVES

Biosurfactants are biomolecules that are produced by many microorganisms. These compounds are amphiphilic i.e., they contain both hydrophilic and hydrophobic moieties. They reduce the surface tension of the medium in which they are applied. This can be a great source for bioremediation. The current study is based on the production of biosurfactant from different bacteria and their effect in the metal bioremediation. To carry out the study the following objectives were taken into consideration.

1. Screening, Production and extraction of biosurfactant from bacteria.
2. Chemical characterization of extracted biosurfactant by FTIR.
3. Biochemical characterization of biosurfactant producing bacteria.
4. Growth kinetics of biosurfactant producing bacteria.
5. Antimicrobial activity of the extracted biosurfactant on pathogenic bacteria.
6. Surface tension determination using tensiometer and metal remediation studies of the isolated biosurfactants.

MATERIALS AND METHODS

1. Isolation of biosurfactant producing bacteria

1.1. Sample collection:

1. Soil samples were collected from Vishakhapatnam from four different sites; zinc industry, Hindustan Petroleum Corporation Limited (HPCL), Andhra Petro Chemicals Limited (APCL) and Vishakhapatnam port.
2. Water samples were collected from Paradeep port located in Odisha.
3. Water samples were collected from Bhitarkanika located in Odisha.

2. Isolation and enumeration of bacterial colonies

5 g of each soil samples were inoculated in 100ml of Mineral Salt Medium (MSM) [NaNO₃ (0.5 g/l), K₂HPO₄ (0.5 g/l), KH₂PO₄ (0.5 g/l), MgSO₄·7H₂O (0.5 g/l), KCl (0.1 g/l) and FeSO₄ (0.01 g/l)] with 1ml petrol added to flasks containing HPCL, APCL and Vishakhapatnam port respectively and 1% of dextrose was added to flask containing zinc industry sample as the sole carbon sources respectively, and all the flasks were incubated for 72 hours at 37°C. The Mineral Salt Medium (MSM) used here was modified from that of Dubey and Juwarkar (2001). The samples then were serially diluted up to 10⁻⁶ dilution. 1 ml of which was transferred to sterile petriplates and over that 20 ml of R2A agar was poured. The plates were inverted and incubated at 25° C, for 48 hours. After incubation morphologically distinct colonies were selected for further studies.

Samples from Paradeep and Bhitarkanika were serially diluted upto 10⁻¹⁰ dilution. The colonies were obtained by spread plate technique. Colonies were obtained at 10⁻⁶ dilution. Enumeration of total heterotrophic bacteria was done. Then the colonies were screened for cadmium and mercury resistance. Cadmium resistant bacteria were obtained by inoculating the strains in 100ppm cadmium. Mercury resistant bacteria were obtained by inoculating the strains in 10ppm mercury.

3. Screening of biosurfactant

The isolated colonies were obtained in pure cultures and tested for their biosurfactant production by the following methods.

3.1. Oil spreading technique

In oil spreading assay (Morikawa *et al.*, 1993), 10 µl of crude oil was added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant were gently placed on the centre of the oil layer. The presence of biosurfactant would displace the oil and a clear zone would form. The diameter of the clearing zone on the oil surface would be visualized under visible light and measured after 30 seconds, which correlates to the surfactant activity, also known as oil displacement activity.

3.2. Blood haemolysis test

The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. These plates were incubated for 48 to 72 hours at 37°C. The plates were then observed and the presence of clear zone around the colonies indicated the presence of biosurfactant producing organisms (Anandaraj and Thivakaran, 2010).

3.3. Bacterial adhesion to hydrocarbons (BATH)

The hydrophobicity of the cells can be measured by BATH assay (Rosenberg *et al.*, 1980). Bacterial cells were washed twice with phosphate buffer salt solution (K₂HPO₄ 16.9g/l, KH₂PO₄ 7.3g/l) with pH 7 and were suspended in it to give an optical density of ~ 0.5 at 600nm. 100µl of crude oil (petrol) was added to 2ml of cell suspension and was vortex shaken for 3 min in test tubes. After shaking, crude oil and aqueous phase were allowed to separate for 1hour. OD of the aqueous phase was then measured at 600nm in a spectrophotometer. Hydrophobicity is expressed as the percentage of cell adherence to crude oil and was calculated as follows:

$$100 * (1 - OD \text{ of the aqueous phase} / OD \text{ of the initial cell suspension}).$$

Three independent determinations were made and the mean values were calculated.

3.4. Drop-collapse test

The bacterial strains were inoculated in mineral salts medium with 0.1% crude oil (petrol) and incubated for 48 hours. Drop collapse test was performed to screen the biosurfactant production (Jain *et al.*, 1991). 2µl of crude oil i.e., petrol was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24 hours. The 48 hour culture was centrifuged at 12,000g for 15mins at 25°C to remove the cells. 5 µl of the supernatant was transferred to the oil-coated well regions and drop size was observed after 1 min with the help of a magnifying glass. The result was considered to be positive when the diameter of the drop was increased by 1mm from that which was produced by distilled water which was taken as the negative control (Youssef *et al.*, 2004).

3.5. Penetration Assay

This assay was developed by Maczek (Maczek *et al.*, 2007) for the screening of biosurfactant; the penetration assay. This assay relies on the contacting of two insoluble phases which leads to a color change.

In this assay, the cavities of a 96 well microplate were filled with 150 µl of a hydrophobic paste made up of oil and silica gel. The paste was covered with 20 µl of oil. 10 µl of a red staining solution (safranin) was added to 90 µl of the supernatant. The coloured supernatant was then placed on the surface of the paste.

4. Extraction of biosurfactants

The cultures were inoculated in 50 ml R2B broth to which 1ml of petrol was added. The cultures were incubated at 25°C for 7 days with shaking conditions. After incubation, the cultures were centrifuged at 5000rpm, 4°C for 30 minutes to remove the bacterial cells. To the supernatant thus obtained, 1M H₂SO₄ was added to adjust the pH at 2. Equal volumes of chloroform: methanol was added in the ratio of 2:1. These mixtures were shaken well to ensure proper mixing and were left overnight for evaporation. White coloured precipitate if seen at the interface between the two liquids proved the presence of biosurfactant.

5. Purification of biosurfactants

The biosurfactant formed was carefully taken out with the help of micropipette and kept in eppendorf tubes. 1ml distilled was added to the eppendorf containing biosurfactant and was thoroughly vortexed to ensure uniform mixing. These were centrifuged at 7000rpm, 4°C

for 30 minutes. The supernatant was discarded and the pellet was allowed to dry for 24 hours. The dry pellet thus obtained was the crude extract of biosurfactant.

6. Antimicrobial activity of biosurfactants

20 ml Muller Hinton Agar media was prepared each for 12 petriplates on each of which 3 wells were made and were named as A, B and C respectively. 6 plates were swabbed with *Klebsiella* and 6 plates were swabbed with *Escherichia coli*. To the wells A, purified biosurfactants were added, to the wells B, diluted biosurfactants (10 fold) were added and to the wells C, distilled water (control) was added. The plates were kept in incubation at 37°C for 24 hours. The presence of clear zone marked the antimicrobial activity of biosurfactant. Three readings of the clear zone diameter were taken for each well and the mean was calculated to determine the actual zone diameter (Rodrigues *et al.*, 2006).

7. Antibigram of the biosurfactants

20ml Muller Hinton Agar was prepared for 12 petriplates each. Two plates were assigned for each strain. All the petriplates were swabbed with pathogenic strain (*Escherichia coli*). All the strains were treated with 6 different antibiotics namely; chloramphenicol, kanamycin, neomycin, tetracycline, gentamicin and ampicilin. The plates were incubated at 37°C for 24 hours. After incubation, the plates were checked for the appearance of zone of inhibition. If the zone of inhibition i.e., a clear zone appeared, then the diameter of the zone was recorded. Three independent readings were taken and the mean was calculated which would be the representation of the actual diameter of the zone of inhibition. Based on the measurement of the zone of inhibition, interpretations were made by referring to the zone size interpretation chart.

8. Growth Kinetics

200µl of freshly inoculated cell cultures were taken individually in each well of 96 well microtiter plate (Tarson, Kolkata, India). Then it was placed in the ELISA Reader (Perkin Elmer) and the optical density (O.D.) was measured at 595nm. The consecutive optical densities were taken regularly at the interval of every one hour for 24 hours. The optical densities were plotted against time to determine the growth curve.

9. Emulsification assay

Emulsification assay was carried out using petroleum (Cooper and Goldenberg, 1987). 2ml of hydrocarbon i.e., petrol was taken in a test tube to which 1ml of cell free supernatant obtained after centrifugation of the culture, was added and was vortexed for 2 minutes to ensure homogenous mixing of both the liquids. The emulsification activity was observed after 24 hours and it was calculated by using the formula:

$$\text{total height of the emulsion} / \text{height of the aqueous layer} * 100$$

The calculations were done for all the cultures individually and their emulsification activities were compared with each other.

10. Physical characterization of bacterial isolates

10.1. Colony morphology

The shape, size, elevation, margin and colour of the colony were observed in the culture plates with Luria Bertani Agar used as the nutrient medium. The observations were noted down.

10.2. Gram Staining

Clean dry glass slides were taken on which smears of diluted bacterial suspensions were made. These smeared slides were heat fixed by using a spirit lamp. Each slide were treated with crystal violet solution and kept for 1 minute. Then the slides were washed with distilled water. The slides were then flooded with Gram's iodine, kept for 1 minute and again washed with distilled water. Then 1-2 drops of Gram's decolourizer was put on the smears and kept for 30 seconds and washed properly with distilled water. Then the counter stain i.e., safranin was applied on the smears and kept for 1 minute and again washed with distilled water. The slides were then air dried and observed under light microscope at 100x objective. The cells were identified to be Gram positive if they retained the violet colour of crystal violet or Gram negative if they appeared pink by retaining the colour of safranin.

10.3. Cell morphology

The Gram stained cells were observed under the light microscope under 100x using oil immersion. The shape and colour of the cells were determined.

10.4. Characterization of biosurfactant producing bacteria by Scanning Electron Microscopy

Smears of bacterial strains were made on glass slides measuring 1x1cm and were treated with 2.5% glutaraldehyde and kept for 10 hours. Then the slides were treated with 1% tannic acid and then washed in 30%, 70% and 90% ethanol for 10 minute each respectively. The slides were then air dried and observed at various resolutions under SEM.

11. Biochemical characterization

HiBacillus and HiCarbohydrate identification kit assay were performed by using HiMedia Rapid Biochemical Identification kit [KB003 Hi25®]. 50µl cultures were taken and added to each wells of the kit and were incubated at 37°C for 24 hours. Then the identification was done from the kit result sheet.

11.3. Motility test

Fresh cultures were taken and inoculated in Mannitol Motility Nitrate Agar and kept in incubation for 24 hours. After incubation, the cultures were checked for motility. If the cultures showed growth along the line of inoculation then the strains are non motile and if they spread all over the medium then the strains are motile.

11.4. Oxidase test

Fresh colonies were streaked on the discs. The change in colour was observed after 10 seconds. If the colour changed from white to purple then the result was considered to be positive and if there was no colour change then the result was considered to be negative.

12. Characterization of biosurfactant

12.1. Fourier Transform Infrared analysis (FTIR)

FTIR spectroscopy was carried out using crude biosurfactant extract obtained from the acid precipitation of the cell free culture supernatant. IR Prestige- 21Fourier Transform Infrared spectrophotometer (Samadzku, Japan) was used to determine the chemical nature of the biosurfactant by the KBr pellet method (Das *et al.*, 2008a, b; Mukherjee *et al.*, 2009).

1.2. Surface tension measurement

Crude biosurfactant adjusted to volume 50ml was taken for the measurement of surface tension with respect to distilled water. The respective surface tensions readings

obtained from tensiometer were plotted for each bacterial strain. The differences in the surface tension with respect to distilled water were hence determined (ABU-Ruwaida *et al.*, 1991).

1.3. Effect of biosurfactant on metal removal

The bacterial cultures were inoculated in Glycerol Mineral Salt Medium (Gly MSM) for 7 days. The 7 days incubated cultures were subjected to centrifugation at 7000 rpm for 20 minutes at 4°C. The supernatant were discarded and the pellet was washed with 0.9% NaCl (normal saline). The pellet obtained was then treated with 50ml lead metal solution (100ppm lead nitrate solution), and incubated for 72 hours at 180 rpm. After incubation the metal solutions were centrifuged at 7000 rpm for 20 minutes at 4°C. The supernatants were analysed for the left over metal by Atomic Absorption Spectroscopy (AAS). The adsorbed by bacterial cells were calculated (Pérez et al., 2007) by:

$$Q=V (C_0-C_e)/X$$

Where,

Q= specific metal uptake (mg/g)

V= volume of metal solution (ml)

C₀= initial concentration of metal in the solution (mg/l)

C_e= final concentration of metal in the solution (mg/l)

X= dry weight of the biomass (g)

12.4. Carbohydrate and protein estimation

Carbohydrate and protein estimation of biosurfactant were done by the following methods. Carbohydrate estimation was done by phenol-sulfuric acid method (Dubois *et al.*, 1956). Protein estimation was done by Bradford method (Bradford, 1976).

RESULTS

1. Enumeration of total viable cell count

The total viable counts of the bacteria were done from the plates having 40 to 400 colonies (Table 2). TVC (total viable count) was calculated by using the formula:

$$THB = \text{no. of colonies} * \frac{\text{dilution factor}}{\text{inoculum}} \text{ size } \left(\frac{CFU}{ml} \right)$$

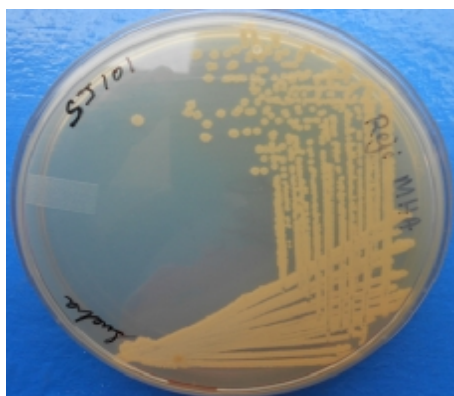
Table 2: Total viable cell count

Strains	No. of bacterial colonies	Dilution factor	THB (CFU/ml)
SJ101	270	10^{-2}	2.7×10^5
	210	10^{-3}	2.1×10^5
	150	10^{-4}	1.5×10^5
SJ102	342	10^{-2}	3.42×10^5
	250	10^{-3}	2.5×10^5
	172	10^{-4}	1.72×10^5
SJ103	283	10^{-2}	2.83×10^5
	225	10^{-3}	2.25×10^5
	170	10^{-4}	1.7×10^5
SJ202	320	10^{-2}	3.2×10^5
	280	10^{-3}	2.8×10^5
	152	10^{-4}	1.52×10^5
SJ204	262	10^{-2}	2.62×10^5
	205	10^{-3}	2.05×10^5
	126	10^{-4}	1.26×10^5
SJ301	250	10^{-2}	2.5×10^5
	172	10^{-3}	1.72×10^5

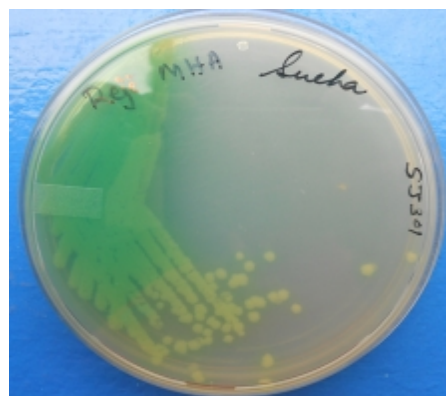
	125	10^{-4}	1.25×10^5
PW03	320	10^{-2}	3.2×10^5
	260	10^{-3}	2.6×10^5
	132	10^{-4}	1.32×10^5
PW05	278	10^{-2}	2.78×10^5
	200	10^{-3}	2.0×10^5
	136	10^{-4}	1.36×10^5
JB201	324	10^{-2}	3.24×10^5
	246	10^{-3}	2.46×10^5
	190	10^{-4}	1.9×10^5
JC101	275	10^{-2}	2.75×10^5
	220	10^{-3}	2.2×10^5
	140	10^{-4}	1.4×10^5

2. Isolation of bacterial colonies

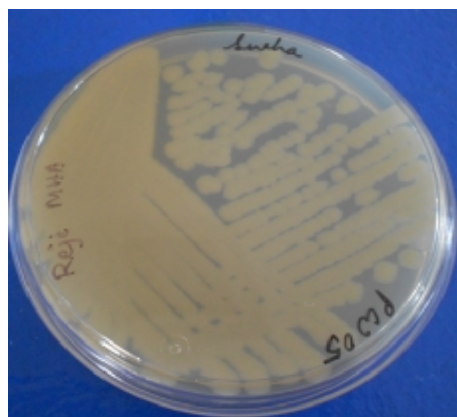
Ten strains observed with different colony morphology were streaked on Muller Hinton Agar plates to obtain pure cultures (Fig 6).



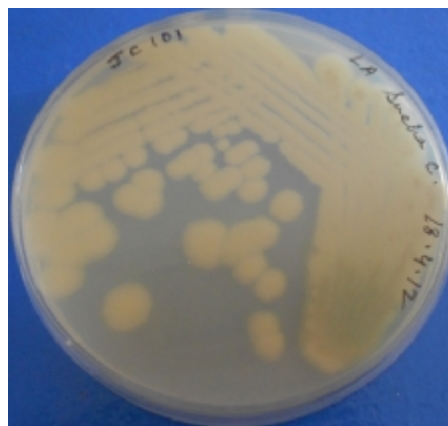
a



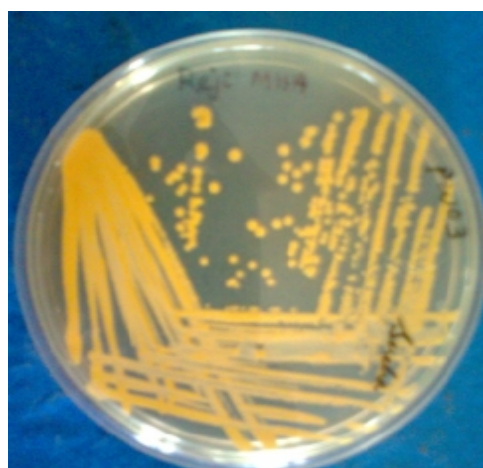
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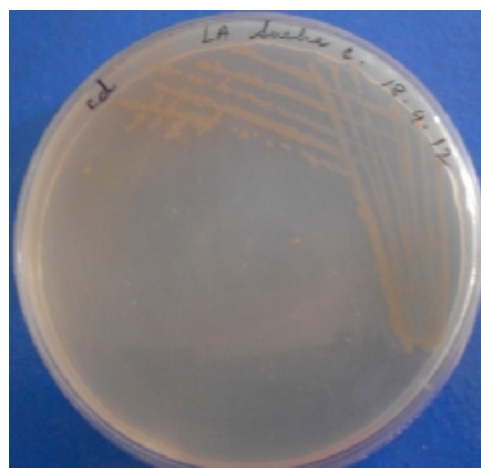
c



d



e



f

Fig 6: Pure cultures of the isolated strains (a - f)

3. Screening of biosurfactant

All the ten isolated strains were screened for biosurfactant production of which the following six strains showed good results and were selected for further study. The strains were named as SJ101, SJ301, JC101, JB201, PW03 and PW05, respectively.

3.1 *Oil spreading technique*

The supernatant of the six strains were added to the plates containing oil. The strain JB202, JC101, PW05, PW03, SJ101 and SJ301 displaced the oil showing a zone of displacement. The organisms which produce biosurfactant can only displace the oil. The results obtained were noted down (Table 3).

Table 3: Oil spreading technique

STRAIN	DIAMETER (in cm)	INTERPRETATION
SJ101	4	NEGATIVE
SJ102	1.2	NEGATIVE
SJ301	5.2	POSITIVE
SJ103	0	NEGATIVE
JC101	6.1	POSITIVE
SJ202	1.7	NEGATIVE
JB202	7	POSITIVE
PW03	4.6	POSITIVE
SJ204	0.6	NEGATIVE
PW05	6.2	POSITIVE

2.1. Blood haemolysis test

All the strains were streaked on blood agar plates. All the six strains showed positive results for haemolytic activity i.e., formation of a clear zone around the colonies (Fig 7).

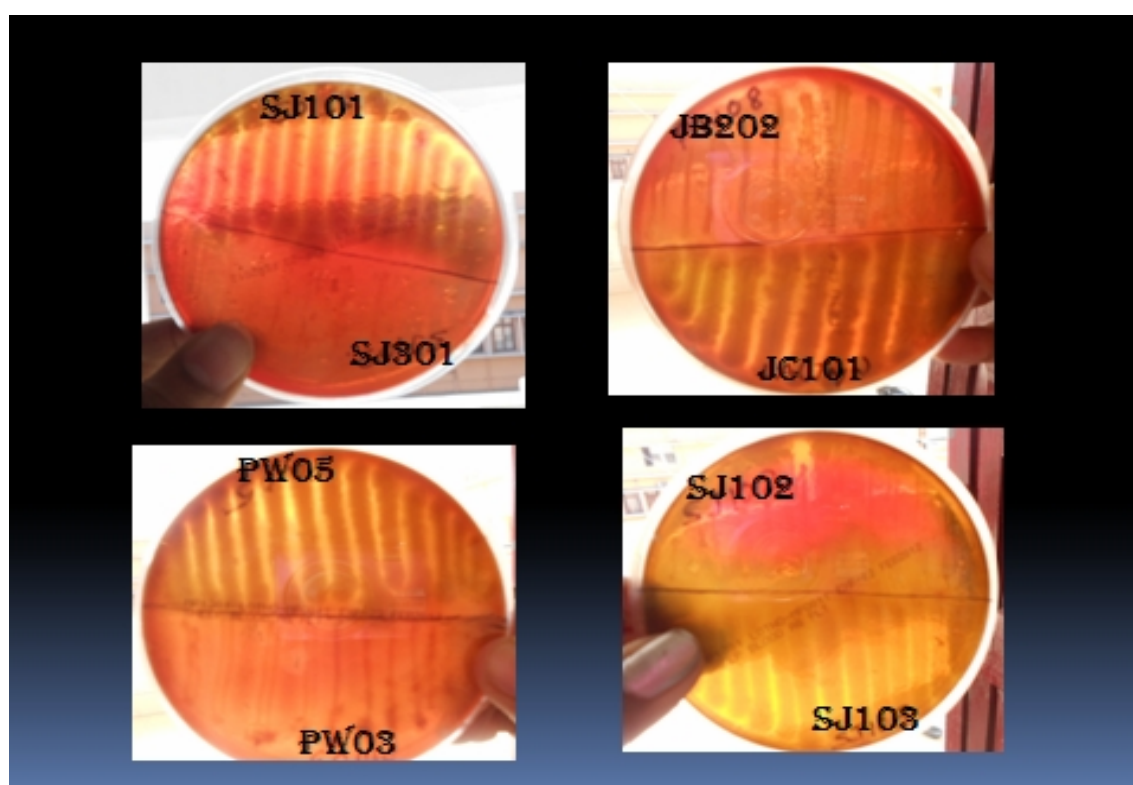


Fig 7: Blood agar plates

3.2 . Bacterial adhesion to hydrocarbons:

In this assay a turbid, aqueous suspension of washed microbial cells was mixed with a distinct volume of a hydrocarbon, petrol. After mixing for 2 minutes, the two phases were allowed to separate. Hydrophobic cells were bound to hydrocarbon droplets and had risen with the hydrocarbon. They were removed from the aqueous phase. The turbidity of the aqueous phase was measured. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cells. The percentage of cells bound to the hydrophobic phase (H) is calculated by:

$$H = (1-A/A_0) * 100$$

Where, A_0 is the absorbance of the bacterial suspension without hydrophobic phase added and A the absorbance after mixing with hydrophobic phase (Table 4).

Table 4: BATH assay readings

Strains	A	A₀	H%
JB202	1.35	1.94	30
JC101	0.49	0.96	49
PW05	0.47	0.75	38
PW03	1.47	1.96	25
SJ301	0.38	0.89	58
SJ101	0.97	1.54	38

3.3 . Drop collapse test:

This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant were placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules were repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface was reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. All the strains tested gave positive results for drop collapse test.

3.4. Penetration assay:

For this assay, the cavities of a 96 well microplate were filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste was covered with 10 µl of oil. Then, the supernatant of the culture was colored by adding 10 µl of a red staining solution to 90 µl of the supernatant. The colored supernatant was placed on the surface of the paste. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste. The silica is entering the hydrophilic phase and the upper phase will change from clear red to cloudy white within 15 minutes. The described effect relies on the phenomenon that silica gel is entering the hydrophilic phase from the hydrophobic paste much more quickly if biosurfactants are present. Biosurfactant free supernatant will turn cloudy but stay red (Table 5).

Table 5: Penetration assay

STRAIN	RESULTS
SJ101	+
SJ301	+
JC101	-
JB202	+
PW03	-
PW05	+

4. Extraction of biosurfactants

The 7 days culture, inoculated in R2B broth with 1ml of petrol, was centrifuged at 5000 rpm at 4°C for 30 minutes to obtain the supernatant. To the supernatant, equal volumes of chloroform: methanol in the ratio 2:1 was added and the mixture was acid precipitated with 1M H₂SO₄. The pH was adjusted to 2 and kept overnight for evaporation. White coloured precipitate was seen at the junction of the two immiscible liquids i.e., supernatant and chloroform: methanol. This white precipitate, which was the biosurfactant, was observed in all the six strains.

5. Antimicrobial activity of biosurfactant

The antimicrobial activity of the biosurfactant produced by each strain was seen for 2 pathogenic bacteria; *Escherichia coli* and *Klebsiella*. It was seen that biosurfactant produced

by SJ301 showed best antimicrobial activity for both *Escherichia coli* (Table 7) and *Klebsiella* (Table 6) by forming the biggest zone of inhibition in comparison with the other strains. SJ101 did not show any antimicrobial activity.

Table 6: Measurement of zone of inhibition for *Klebsiella*

STRAIN	A	Mean of A (cm)	B	Mean of B (cm)	Control
SJ101	-	-	-	-	-
	-		-		-
	-		-		-
SJ301	2.6	2.56	2.1	2.06	-
	2.5		2.1		-
	2.6		2.0		-
PW03	1.5	1.4	1.2	1.2	-
	1.4		1.2		-
	1.3		1.2		-
PW05	-	-	-	-	-
	-		-		-
	-		-		-
JC101	2.4	2.26	1.2	1.2	-
	2.1		1.1		-
	2.3		1.3		-
JB201	2.5	2.43	2.1	2.03	-
	2.4		2.1		-
	2.4		1.9		-

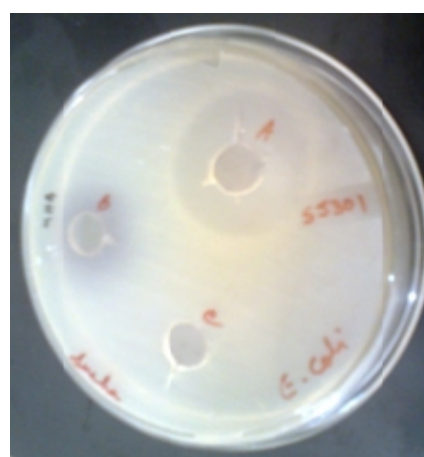
Table 7: Measurement of zone of inhibition for *Escherichia coli*

STRAIN					
SJ101	-	-	-	-	-
	-		-		-
	-		-		-
SJ301	3.7	3.6	1.8	1.76	-
	3.5		1.7		-
	3.6		1.8		-

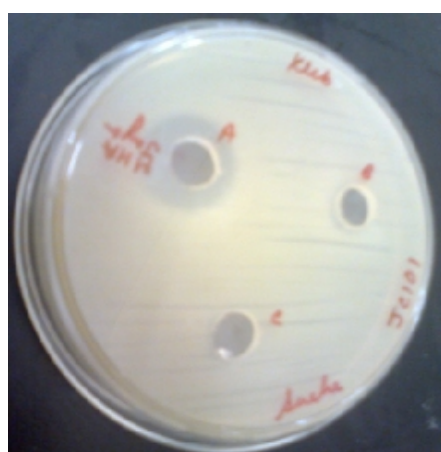
PW03	1.0	1.16	0.6	0.4	-
	1.2		0.5		-
	1.3		0.4		-
PW05	1.1	1.1	0.3	0.26	-
	1.0		0.2		-
	1.2		0.3		-
JC101	2.3	2.23	1.5	1.3	-
	2.1		1.1		-
	2.3		1.3		-
JB201	2.1	2.43	1.4	1.8	-
	2.4		2.1		-
	2.4		1.9		-



A. SJ301 for *Klebsiella*



B. SJ301 for *Escherichia coli*



C. JC101 for *Klebsiella*



D. PW05 for *Klebsiella*

Fig 8: Antimicrobial activity of biosurfactants (A-D)

6. Antibiotic sensitivity test for biosurfactant producing bacteria

All the strains which produced biosurfactant were checked for their sensitivity towards different antibiotics namely chloramphenicol, kanamycin, neomycin, tetracycline, gentamicin and ampicillin by the appearance of a clear zone around the antibiotic discs. The diameter was measured 3 times and their means were checked with zone size interpretation chart. It was seen that SJ101 showed maximum sensitivity towards chloramphenicol, SJ301 showed maximum sensitivity towards gentamicin, PW03 showed maximum sensitivity towards chloramphenicol, PW05 showed maximum sensitivity towards chloramphenicol, JC101 showed maximum sensitivity towards chloramphenicol and JB201 showed maximum sensitivity towards gentamicin. Antibiotic to which most of the strains were resistant was ampicillin (Table 8).

Table 8: Antibiotic sensitivity test of biosurfactant producing bacteria

Strain	Antibiotics	Diameter (cm)			Mean diameter (cm)	Interpretation
SJ101	Chloramphenicol	3.5	3.7	3.6	3.6	C ^S
	Kanamycin	2.9	3.0	3.0	2.96	K ^S
	Neomycin	2.4	2.5	2.6	2.5	N ^S
	Tetracycline	2.9	2.8	2.7	2.8	T ^S
	Ampicilin	3.4	3.5	3.4	3.43	Am ^S
	Gentamicin	3.2	2.9	3.2	3.1	Gen ^S
SJ301	Chloramphenicol	2.0	1.8	2.0	1.93	Intermediate
	Kanamycin	1.1	1.2	1.2	1.16	K ^R
	Neomycin	2.1	2.1	2.2	2.13	N ^S
	Tetracycline	1.3	1.5	1.8	1.53	Intermediate
	Ampicilin	-	-	-	-	Am ^R
	Gentamicin	2.8	2.8	2.7	2.76	Gen ^S
PW03	Chloramphenicol	4.3	4.2	4.2	4.23	C ^S
	Kanamycin	2.4	2.4	2.4	2.4	K ^S
	Neomycin	3.1	3.0	3.0	3.03	N ^S
	Tetracycline	3.3	3.2	3.1	3.2	T ^S
	Ampicilin	2.2	2.3	2.2	2.23	Am ^S
	Gentamicin	3.2	3.2	3.1	3.16	Gen ^S

PW05	Chloramphenicol	2.7	2.8	2.7	2.73	C ^S
	Kanamycin	2.1	2.0	2.2	2.1	K ^S
	Neomycin	2.5	2.3	2.4	2.4	N ^S
	Tetracycline	2.3	2.6	2.5	2.46	T ^S
	Ampicilin	-	-	-	-	Am ^R
	Gentamicin	2.2	2.6	2.5	2.43	Gen ^S
JC101	Chloramphenicol	2.5	2.5	2.7	2.56	C ^S
	Kanamycin	2.2	2.2	2.2	2.2	K ^S
	Neomycin	2.4	2.4	2.3	2.36	N ^S
	Tetracycline	2.4	2.4	2.3	2.36	T ^S
	Ampicilin	-	-	-	-	Am ^R
	Gentamicin	2.5	2.5	2.4	2.46	Gen ^S
JB201	Chloramphenicol	2.3	2.3	2.4	2.33	C ^S
	Kanamycin	1.6	1.6	1.7	1.63	Intermediate
	Neomycin	2.0	2.0	1.9	1.96	N ^S
	Tetracycline	1.7	1.3	1.6	1.53	Intermediate
	Ampicilin	-	-	-	-	Am ^R
	Gentamicin	2.7	2.6	2.7	2.66	Gen ^S

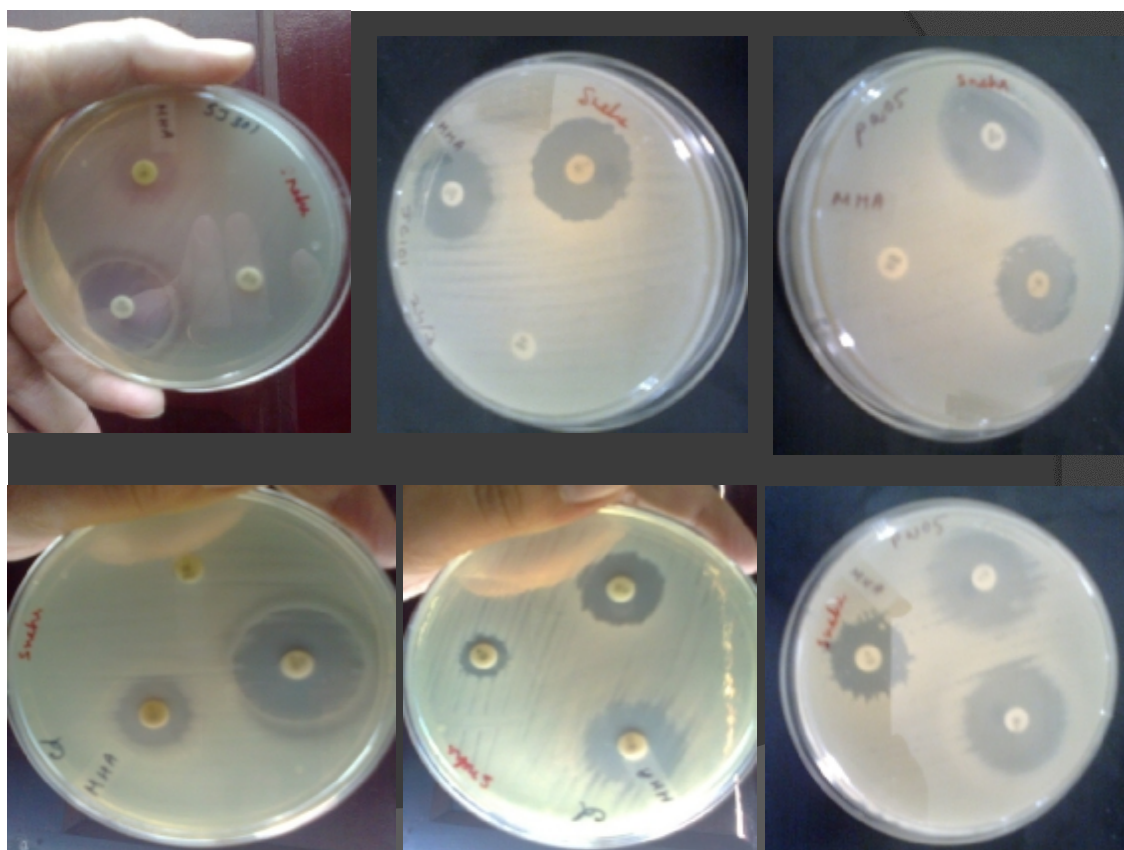
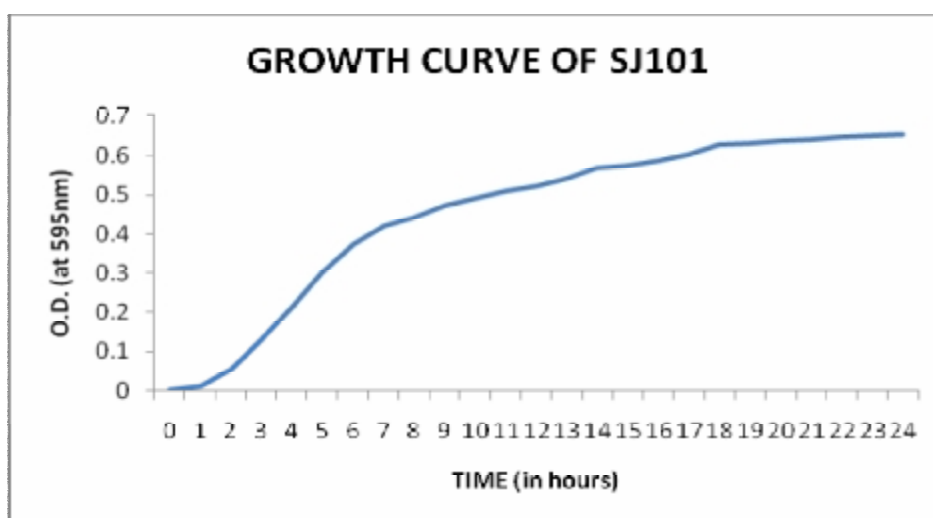


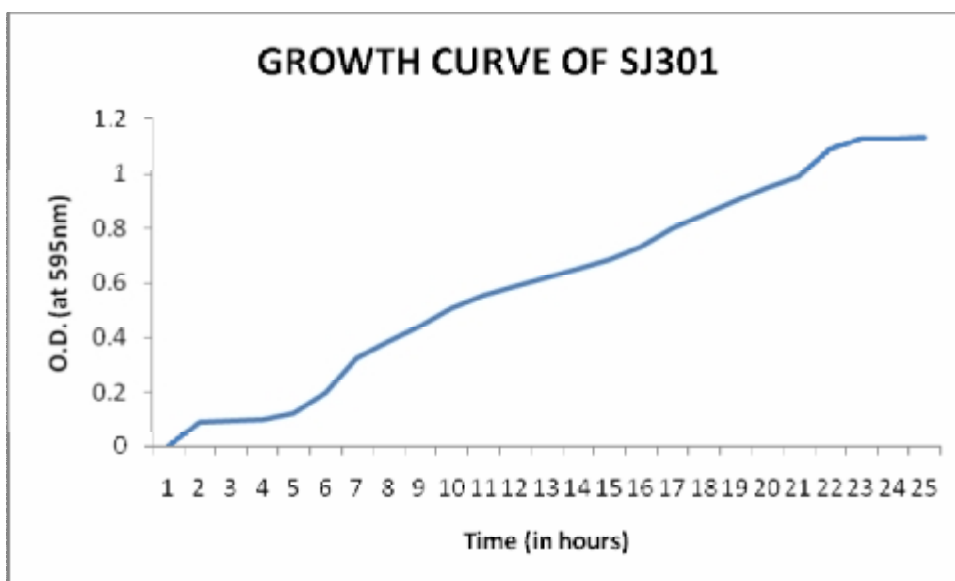
Fig 9: Antibiogram of the isolated strains PW05, PW03, SJ101, SJ301, JC101, JB201

7. Growth kinetics

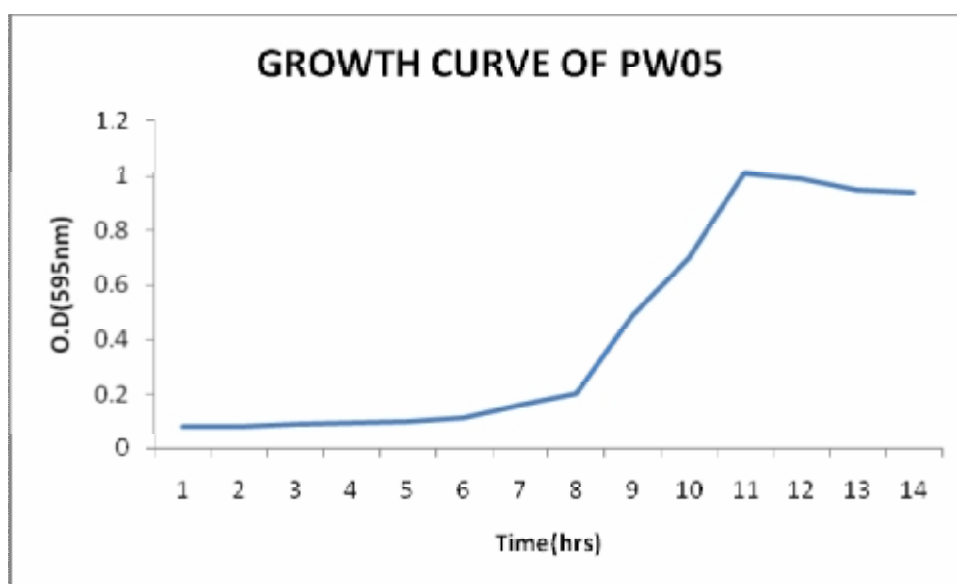
Freshly inoculated cultures were checked for their growth kinetics. Optical densities were measured in ELISA reader (Perkin Elmer). The readings were plotted against time from 0 hour to 24 hours at an interval of every 1 hour at 595nm (Fig10).



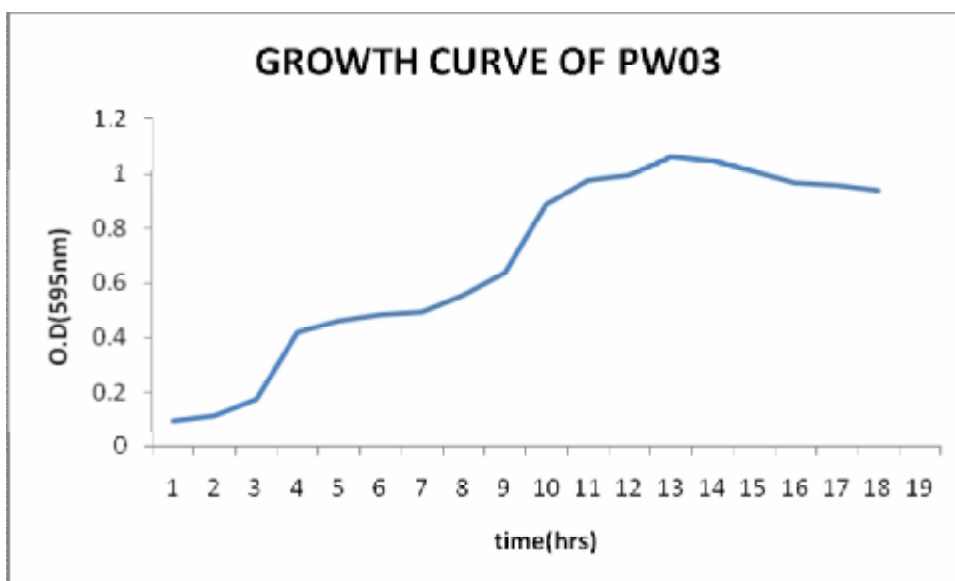
(a)



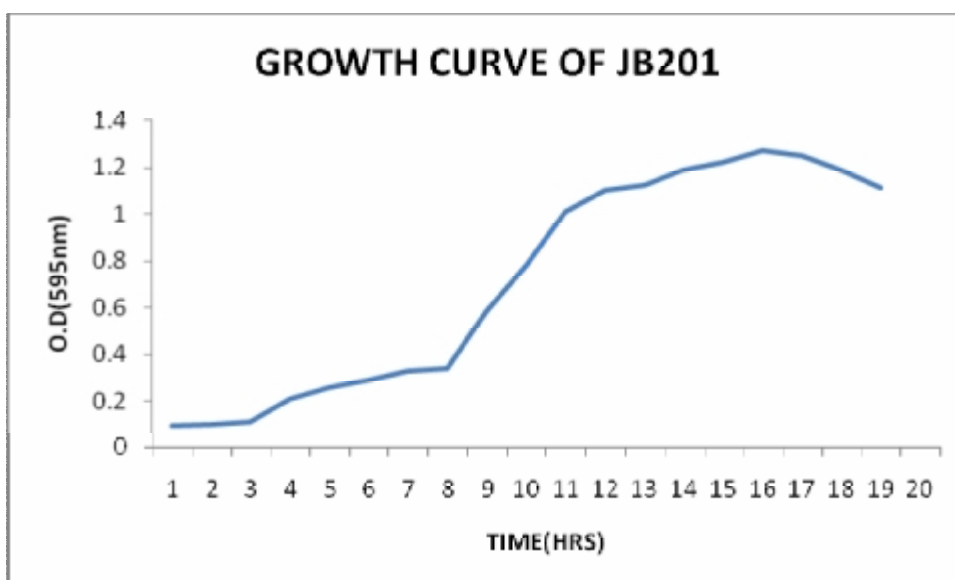
(b)



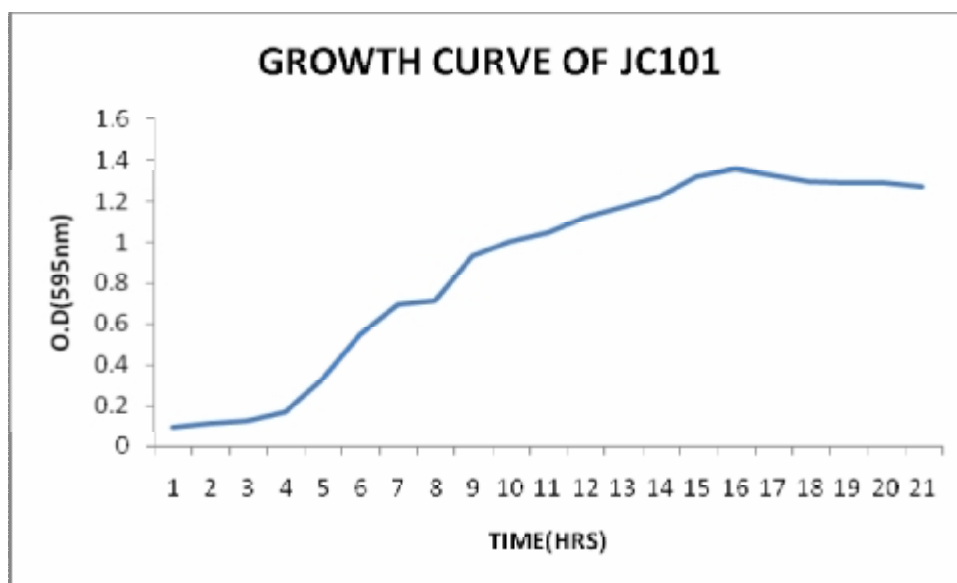
(c)



(d)



(e)



(f)

Fig 10: (a-f) Growth curves of all the strains

8. Emulsification assay

The isolated bacteria were checked for their abilities to emulsify crude oil i.e., petrol in this case. Screening was done by adding 2ml of petrol to 1ml of culture supernatant and kept overnight. The emulsification activities were calculated in terms of percentage (Table no.9). It was seen that the strain PW03 showed the maximum emulsification activity (61.11%).

Table 9: Emulsification activity of all the strains

STRAINS	EMULSIFIED LAYER (in cm)	TOTAL AQUEOUS LAYER (in cm)
SJ101	1	1.9
SJ301	0.9	1.7
PW03	1.1	1.8
PW03	0.8	1.7
JB201	0.9	1.6
JC101	0.8	1.7

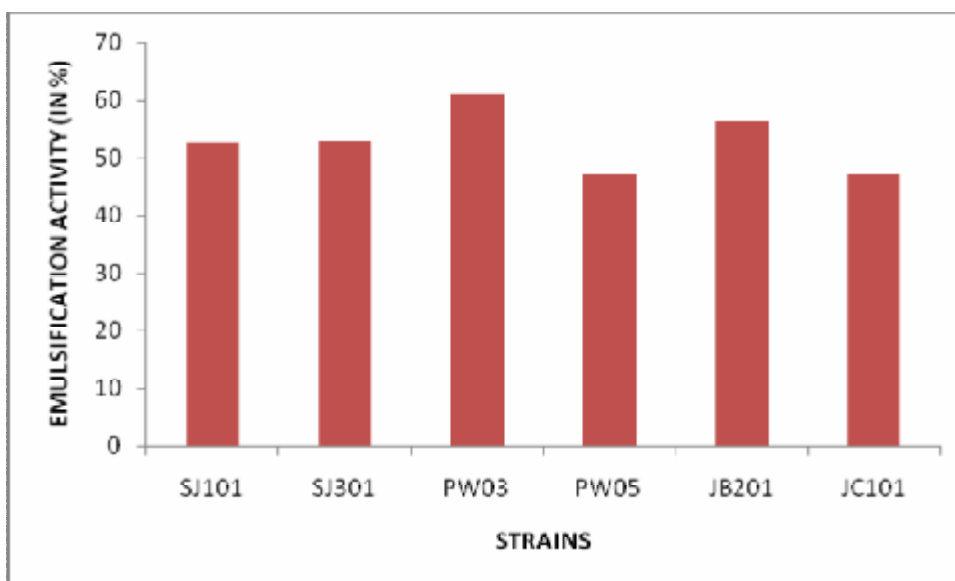


Fig 11: Comparison of emulsification activities of all the strains

9. Physical characterization of bacterial isolates

9.1. *Gram's staining*

Morphological characterizations of bacterial isolates were done by Gram's staining. JC101 was found to be Gram +ve *Streptococcus*. JB201 was found to be Gram –ve *Bacillus* (small). PW05 was found to be Gram +ve *Bacillus*. PW03 was found to be Gram –ve Coccus. SJ301 was found to be Gram +ve *Bacillus* (short). SJ101 was found to be Gram +ve *Bacillus* (Fig 12).

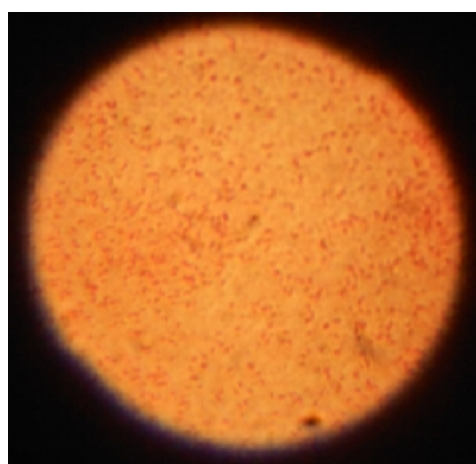


Fig 12.1: JB201 small Gram –ve coccus

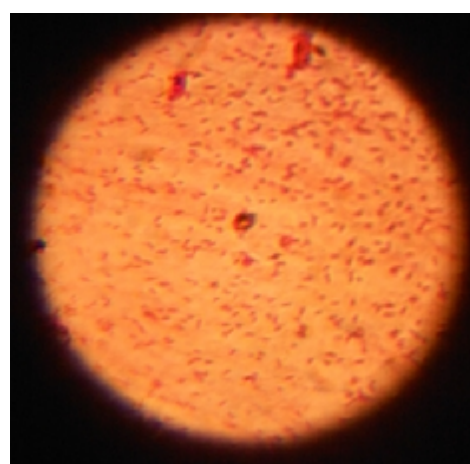


Fig 12.2: SJ301 Gram +ve coccus

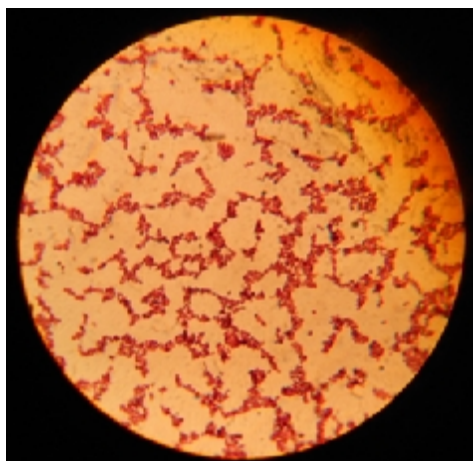


Fig 12.3: PW03 Gram -ve Coccus

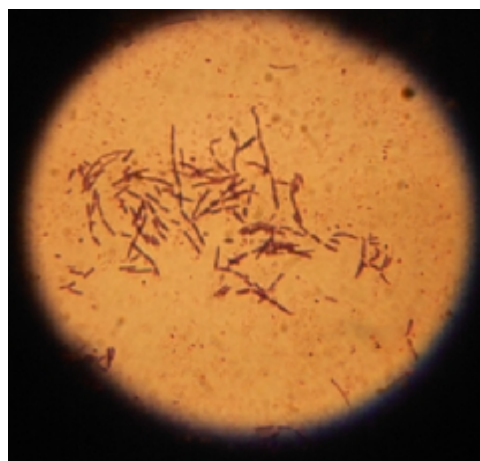


Fig 12.4: PW05 Gram +ve *Bacillus*

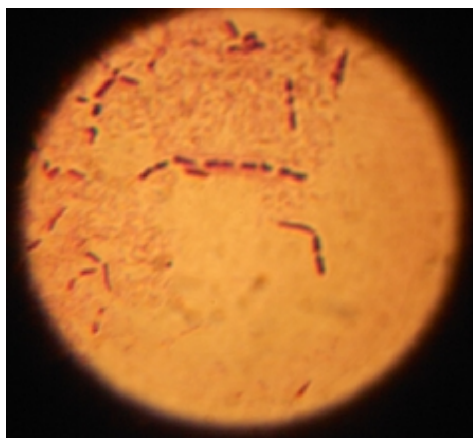


Fig 12.5: JC101 Gram +ve *Bacillus*

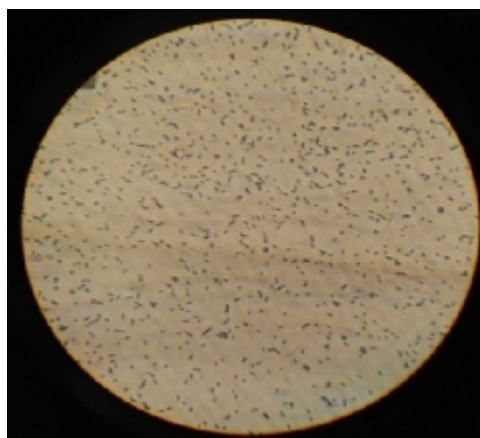
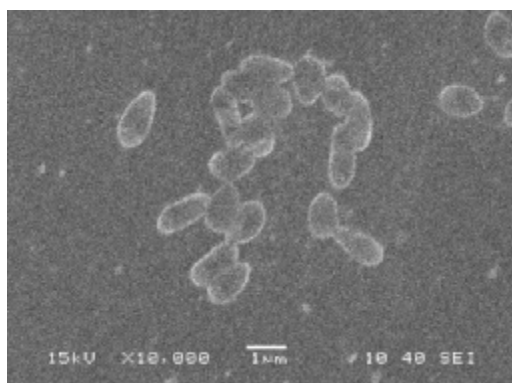


Fig 12.6: SJ101 Gram +ve *Bacillus*

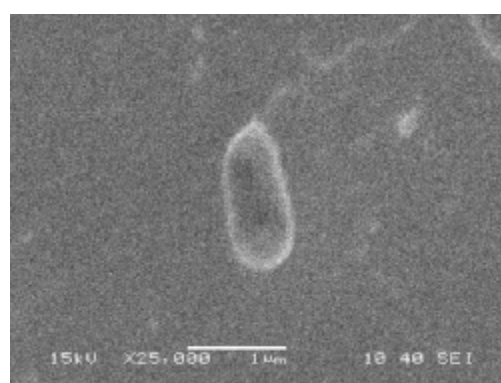
Fig 12: Gram staining pictures

9.2. Scanning electron microscopy

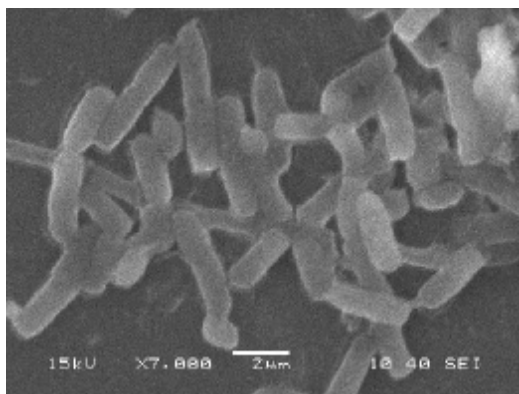
SEM images of the strains JB201, JC101, PW05 and PW03 are given below (Fig 13).



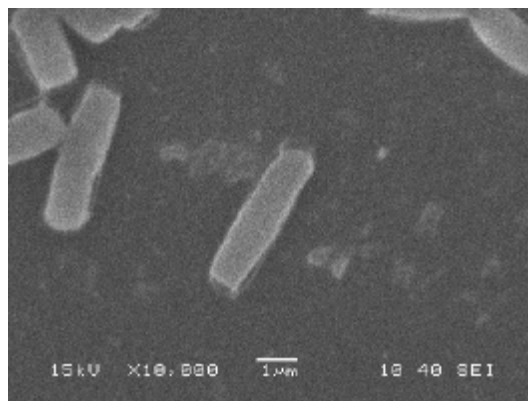
(a) JB201



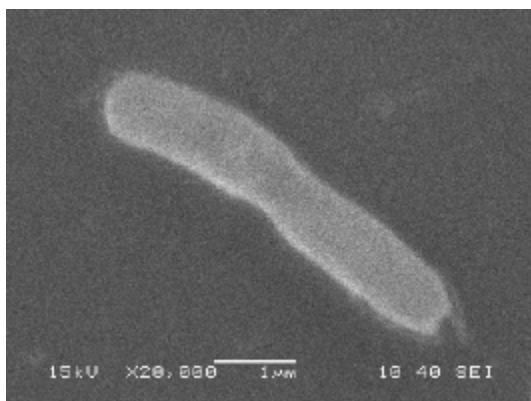
(b) JB201



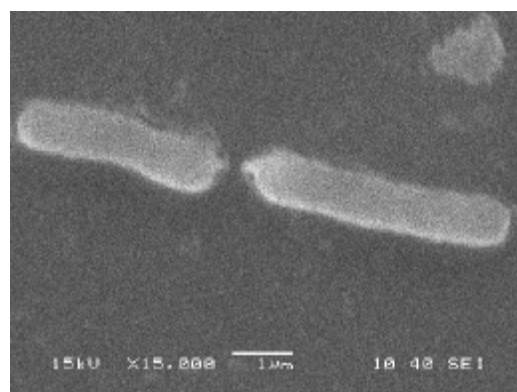
(c) JC101



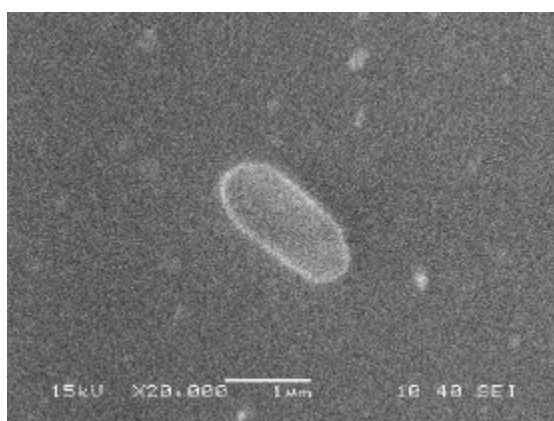
(d) JC101



(e) PW05



(f) PW05



(g) SJ301

Fig 13: SEM Images of JB201 (a, b), JC101 (c, d), PW05 (e,f) and PW03 (g)

10. Biochemical characterization

10.1. *HiBacillus and HiCarbohydrate assay*

By observing the change in colour in the kit to which 50µl of culture were given the following observations were made (Table 10 and Table 11).

Table 10: HiBacillus biochemical result

trains	Malonate	Voges Proskauer's	Citrate	ONPG	Nitrate reduction	Catalase	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalo
J101	-	-	+	-	+	+	+	NR	NR	NR	-	-
J301	+	-	+	-	-	+	+	-	-	-	+	-
W03	-	-	+	-	+	+	-	+	+	+	+	+
W05	+	-	+	-	+	+	+	-	-	+	-	+
C101	+	-	+	-	+	+	+	-	-	NR	-	+
B201	+	-	+	-	-	+	+	-	-	-	-	-

- (Negative), + (positive), NR (not reported)



a) JC101



b) SJ101



c) PW05



d) Control

Fig 14: HiBacillus result (a-d)

Table 11: HiCarbohydrate biochemical result

Sl. No.	Test	Strain (JB201) interpretation
1	Lactose	-
2	Xylose	+
3	Maltose	-
4	Fructose	-
5	Dextrose	-
6	Galactose	+
7	Raffinose	+
8	Trehalose	-
9	Melibiose	-
10	Sucrose	-
11	L-arabinose	+
12	Mannose	-
13	Inulin	-
14	Sodium gluconate	-
15	Glycerol	-
16	Salicin	-
17	Dulcitol	-
18	Inositol	-
19	Sorbitol	-
20	Mannitol	-
21	Adonitol	-
22	Arabitol	-
23	Erythritol	-
24	α -methyl-D-glucoside	-
25	Rhamnose	-
26	Cellobiose	-
27	Melezitose	-
28	α -methyl-D-mannoside	-
29	Xylitol	+
30	ONPG	-
31	Esculin hydrolysis	+
32	D-arabinose	-
33	Citrate utilization	+
34	Malonate utilization	+
35	Sorbose	-

- (Negative), + (positive)



Fig 15: HiCarbohydrate result for JB201

10.2. *Motility test*

Motility test was performed for all the strains in Mannitol Motility Nitrate Agar to check for the motile bacteria. It was seen that only SJ301 was motile showing +ve result and all the other strains were non-motile (Fig 16).



Fig 16: Motility test result (-ve, +ve, -ve)

10.1 *Oxidase test*

Oxidase test was performed with all the strains. It was seen that strains SJ101, SJ301, PW03 and JB201 showed +ve result by changing the colour of the discs from white to violet. PW05 and JC101 showed -ve result (Fig 17).



Fig 17: Oxidase test result (SJ101, SJ301, PW05, PW03, JC101, JB201)

11. Fourier Transform Infrared analysis (FTIR):

FTIR spectral overlap of the isolated biosurfactant occurs at a characteristic vibrational shift from 2000 – 2500 cm^{-1} , more specifically indicating phosphine and isocyanate functional group, in both of the isolates JC101 and SJ301 (Fig 18 & Fig 19).

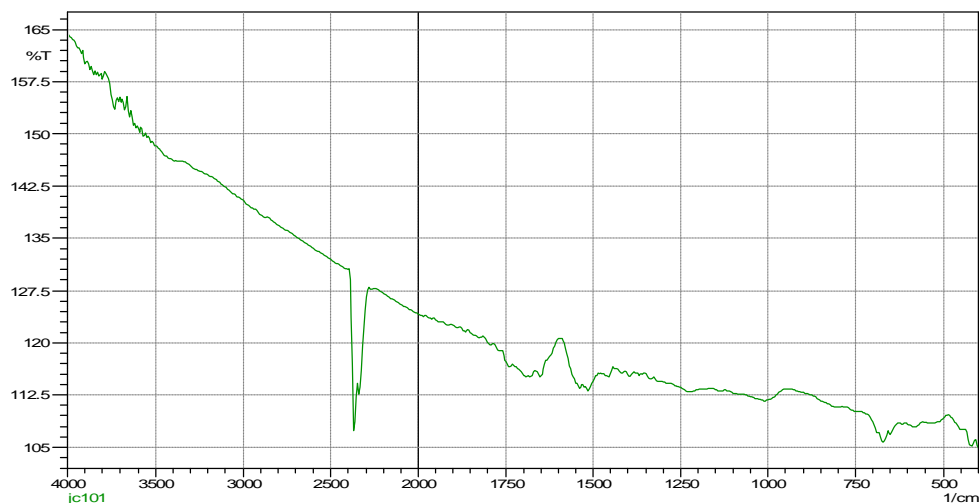


Fig 18: FTIR result for JC101

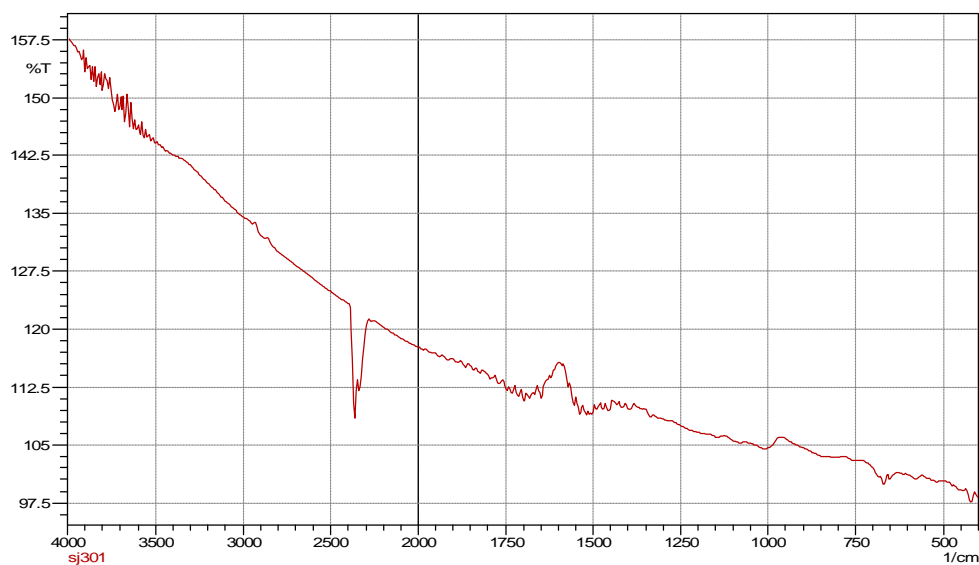


Fig 19: FTIR result for SJ301

12. Surface tension measurement

Surface tension of crude biosurfactant was determined by a tensiometer with respect to distilled water. It was seen that SJ301 showed the maximum reduction in surface tension as compared to others (Fig 20).

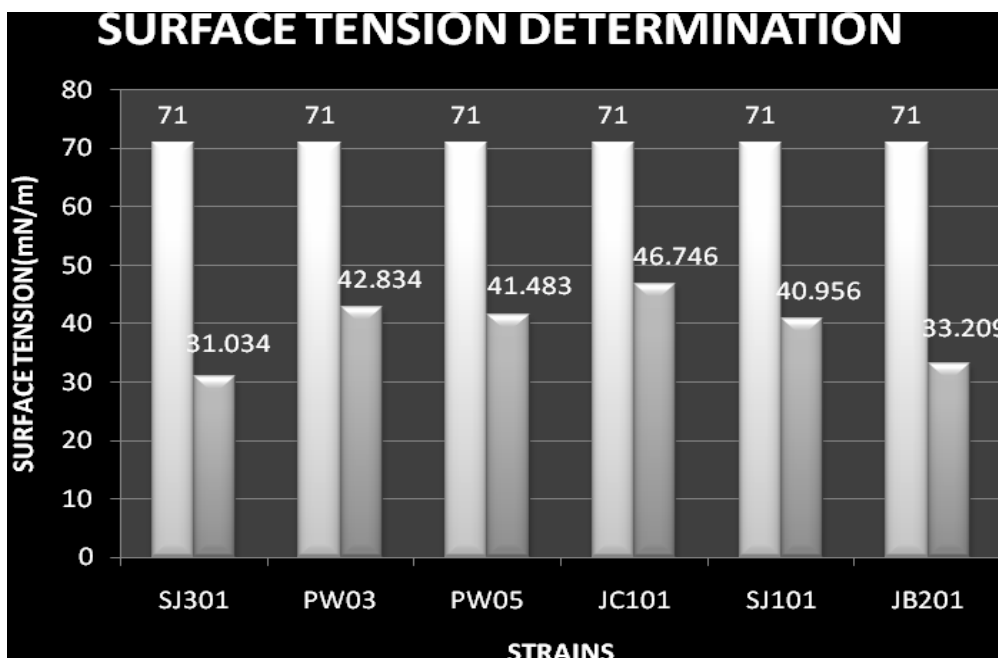


Fig 20: Surface tension determination of the biosurfactants

13. Atomic Absorption study for lead removal by SJ301

The standard curve (Fig 21) and the amount of metal absorbed (mg/g) are given below. The maximum amount of metal absorbed (removed from the medium) is 2.67 mg/g (Table 12).

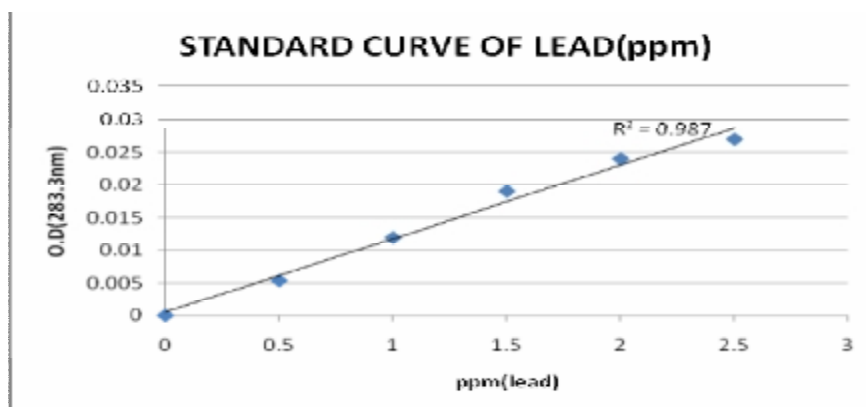


Fig: 21: Standard curve of lead (ppm)

Table 12: AAS readings

1	Conc.(ppm)	O.D(283.3nm)	Metal Absorbed(mg/g)
2	50	6.12	0
3	48.13	5.89	0.493
4	45.01	5.51	0.495
5	42.97	5.26	0.501
6	41.66	5.17	0.599
7	40.03	4.9	0.711
8	39.9	4.88	1.002
9	39.87	4.86	2.67

14. Carbohydrate and Protein estimation

The standard curve of carbohydrate (Fig 22) and the amount of carbohydrate content in the biosurfactants is shown. The maximum carbohydrate content was observed in the strain PW03 (Table 13).

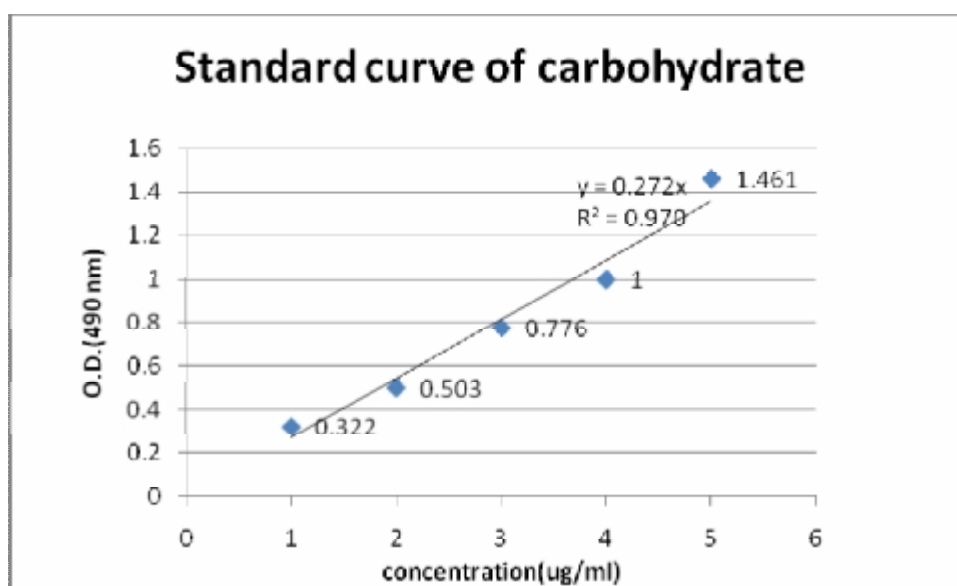


Fig 22: Standard curve of carbohydrate

Table 13: Carbohydrate concentration of the biosurfactants

Strain	O.D(490nm)	Conc. (in 10 ³ mg/ml)
SJ101	1.452	0.39
JC101	0.324	0.088
SJ301	1.550	0.42
PW03	2.298	0.625
PW05	0.567	0.154
JB201	2.119	0.570

The standard curve of protein (BSA) (Fig 23) and the amount of protein content in the biosurfactants is shown. The maximum carbohydrate content was observed in the strain SJ101 (Table 14).

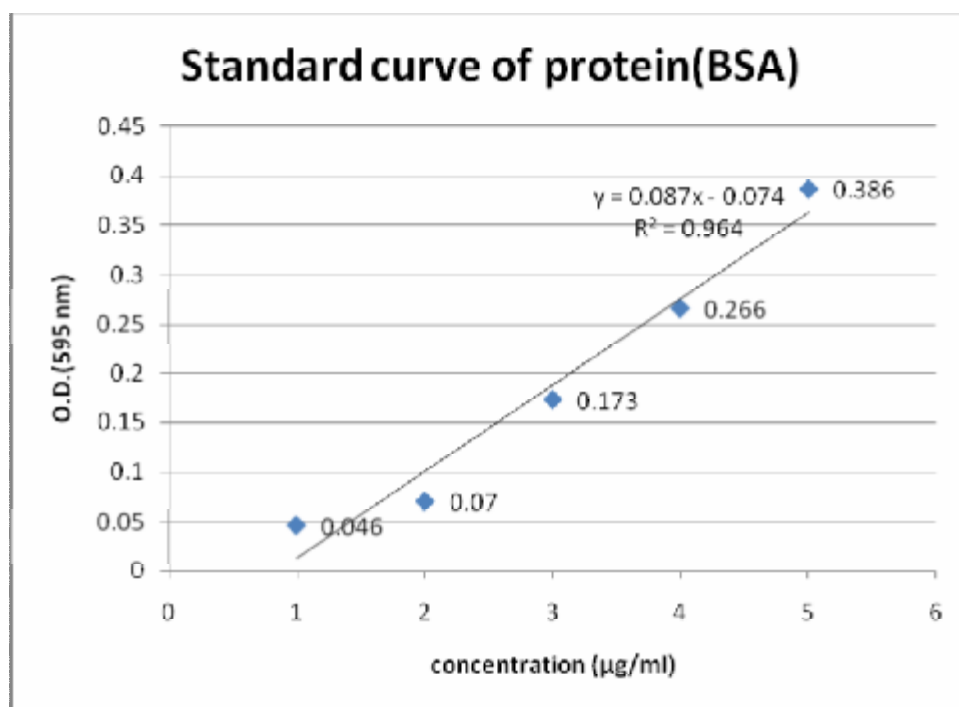


Fig 23: Standard curve of protein

Table 14: Protein concentration of the biosurfactants

Strain	O.D(490nm)	Conc. (in 10 ³ mg/ml)
SJ101	0.226	0.019
JC101	0.183	0.015
SJ301	0.158	0.013
PW03	0.177	0.015
PW05	0.171	0.014
JB201	0.167	0.014

DISCUSSION

The biosurfactant producing bacterial isolates screened from petrochemical wastes and from heavy metal contaminated marine origin showed very good tension-active and emulsifying activities. From oil displacement test (Anandaraj, 2010), drop collapse (Jain *et al.*, 1992), BATH assay (Rosenberg *et al.*, 1980) and emulsification assay (Cooper and Goldenberg, 1987) of the isolated bacteria SJ101, SJ301, JC101, JB201, PW03, PW05 were shown to have high emulsifying activity. The antimicrobial properties of the biosurfactants (Rodrigues *et al.*, 2006) have been widely reported. However, the biosurfactants with antimicrobial properties reported till date is produced mostly by the micro-organisms of terrestrial origin. The oceans cover more than 70% of the Earth's total surface and supports very rich and diverse microflora. However, the number of reports on marine antimicrobial biosurfactant molecules is negligible. Although there have been few reports of marine biosurfactant producers, their antimicrobial potentials have not been explored in details. This problem was identified in the present work and the biosurfactant isolated from marine bacteria as well as petrochemical wastes was tested for antimicrobial action against a battery of pathogenic test organisms. Our results illustrated antimicrobial activity and thus can be useful in many domestic and commercial uses. The antibiogram of the isolated strains secreting biosurfactants were shown to have diverse antibiotic resistance profiles, indicating their diversity and susceptibility in different conditions. The isolated biosurfactant none selectively showed activity against both Gram-positive and Gram negative bacterial strains. This is quite contrasting to earlier reports on antimicrobial actions of the biosurfactants where the lipopeptide biosurfactants have been reported to be active mostly against Gram-positive bacteria (Kitamoto *et al.*, 1993; Singh and Cameotra, 2004). The growth kinetics of the biosurfactant producing isolates was usually between 16-20 hrs having good biosurfactant production during this time period. The chemical characterization of the produced biosurfactant using FTIR showed that that the peak obtained through this analysis usually corresponds to phosphine and isocyanate functional groups, indicating the bacteria isolated to be in *Bacillus* group. The carbohydrate estimation using phenol sulphuric acid test (Dubois *et al.*, 1956) and protein estimation using Bradford assay (Bradford, 1976) showed the carbohydrate and protein content in the extracted biosurfactant. Biochemical characterization of the isolated strains, especially SJ101, PW03, and JC101 gave an unusual profile of Carbohydrate utilisation and thus corresponds to new genera of bacteria. The other three strains showed somewhat similarity to *Bacillus* species. The rationale behind biosurfactant

production upon hydrocarbon (petrol) utilisation should stimulate itself by enhancing the substrate availability. Biosurfactant production using petrol as a carbon source showed better production of biosurfactants. Biosurfactants usually lowers the tensioactive force between the two phases (ABU-Ruwaida *et al.*, 1991). The surface tension of this fraction of the strain SJ301 was found to be the lowest (31.034mNm) indicating its powerful surface tension-reducing property. Biosurfactant utilised in bioremediation has been harnessed relentlessly for biotechnological purposes. Our stain SJ301 having high surface tension reducing property were used for lead removal studies , indicating that this strain was able to remove nearly 2-3% lead from the highly toxic 100 ppm Lead solutions. Thus the strains screened from different origins were efficient producers of biosurfactant having high surface tension reducing ability, antimicrobial activity, and metal remediation property.

CONCLUSION

The interest for biosurfactant production is increasing day by day as it can be used for bioremediation. It is an amphiphilic compound i.e., it has both hydrophilic and hydrophobic moiety. It is produced by a wide range of microorganisms. It is either synthesized on the living cells or is secreted as an extracellular material to reduce the interfacial tension between the surfaces and interface respectively. Bacteria when present in a hydrophobic environment, it is unable to utilize the nutrients from it. By producing biosurfactant in such a condition, converts the hydrophobic layer into small micelles which it can easily engulf as a carbon source which is the basic nutritional requirement.

Crude oil is a very good source of energy both for domestic as well as industrial purposes. Many nations have developed economically by the production of crude oil. Day by day the demand for oil is also increasing in different fields such as industries, automobiles, households, etc. But it is also one of the major pollutants. It forms oil sludge which is entrapped within the effluents. It causes serious problems in the environment. The hydrocarbons in the sludge penetrate from the top soil into the subsoil slowly, presenting a direct risk of contamination to subsoil and groundwater. On the other hand, the light hydrocarbons in the oil sludge vaporize, leaving behind a layer of oil containing dust of soil which blows upwards to pollute the air. Therefore, the oil sludge should be treated to prevent harm to environment. Although burning of the sludge may be simple and easily adaptable, this technique has undesirable hazard in air pollution. Eco-friendly technologies must be used to clean the environment such as degradation by microorganisms. Bioremediation has been accepted as an important method for the treatment of oil pollution by biosurfactant produced by bacterial colonies. Under certain conditions, living microorganisms primarily bacteria can metabolize various classes of hydrocarbons compound. Since hydrocarbons contain high organic matter, it can be assimilated by the bacteria as a carbon source.

Contamination of soil environments with heavy metals is very hazardous for human and other living organisms in the ecosystem. Due to their extremely toxic nature, presence of even low concentrations of heavy metals in the soils has been found to have serious consequences. Nowadays, there are many techniques used to clean up soils contaminated with heavy metals. Remediation of these soils includes non-biological methods such as excavation, and disposal of contaminated soil to landfill sites or biological techniques. Biological methods are processes that use plants (phytoremediation) or microorganisms (bioremediation) to remove metals from soil. Heavy metals are not biodegradable; they can

only be transferred from one chemical state to another, which changes their mobility and toxicity. Microorganisms can influence metals in several ways. Using biosurfactants have unquestionable advantages because bacterial strains able to produce surface active compounds do not need to have survival ability in heavy metal-contaminated soil. The usefulness of biosurfactants for bioremediation of heavy metal contaminated soil is mainly based on their ability to form complexes with metals. The anionic biosurfactants create complexes with metals in a nonionic form by ionic bonds. These bonds are stronger than the metal's bonds with the soil and metal-biosurfactant complexes are desorbed from the soil matrix to the soil solution due to the lowering of the interfacial tension. The cationic biosurfactants can replace the same charged metal ions by competition for some but not all negatively charged surfaces (ion exchange). Metal ions can also be removed from soil surfaces by the biosurfactant micelles.

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